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Nuclear Transplantation: Intracellular Release and Extracellular Influx of Calcium in Response to Electrofusion Pulse and Its Effect on Murine Oocyte Activation and Embryonic Development.

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NUCLEAR TRANSPLANTATION: INTRACELLULAR RELEASE AND EXTRACELLULAR INFLUX OF CALCIUM IN RESPONSE TO ELECTROFUSION PULSE AND ITS EFFECT ON MURINE OOCYTE ACTIVATION AND EMBRYONIC DEVELOPMENT

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ABSTRACT

The transplantation of early embryonic cell nuclei into oocytes in which the maternal chromosomes have been removed is a method by which multiple offspring of the same genotype can be produced. One critical factor required for successful development of nuclear transfer derived embryos is inducing the release of the recipient ooplasm from meiotic arrest (activation). This is accomplished during fusion of the two cells. The DC electrofusion pulse used to fuse the two cells together also activates the recipient ooplasm to resume meiotic events, however, the efficiency of electrofusion pulse induced activation remains low. It is known that activation of the oocyte at fertilization is associated with an increase in intracellular calcium concentration ($[Ca^{2+}]_i$). A series of experiments were designed to look at changes in $[Ca^{2+}]_i$ in murine oocytes after application of a DC electrofusion pulse and its effect on oocyte activation and embryonic development. Oocytes and pronuclear embryos were subjected to the electrofusion pulse in six fusion media consisting of nonelectrolyte (0.3 M mannitol) and electrolyte (phosphate buffered saline, PBS) media containing 0.0, 0.05 or 0.9 mM Ca^{2+}. Significantly ($P<0.01$) higher $[Ca^{2+}]_i$ levels were observed in oocytes pulsed in media containing 0.9 mM Ca^{2+}. An experiment was then designed to test the effect of the three levels of Ca^{2+} on oocyte activation. Oocytes pulsed in media containing 0.9 mM Ca^{2+} exhibited significantly higher rates of activation ($P<0.01$). When 25 μM inositol 1,4,5-triphosphate (IP3) was added to Ca^{2+}-free media, a series of $[Ca^{2+}]_i$ oscillations similar to that found in fertilized oocytes, was observed. Additional experiments were designed to test the rate of fusion of 2-cell embryos subjected to the six fusion media. Significantly higher rates of fusion were obtained in PBS medium

than 0.3 M mannitol ($P<0.05$). The fusion treatments were then applied to pronuclear stage embryos to determine the effect on in vitro development. No difference in rate of development was observed between fusion media ($P>0.05$). In addition, no difference in in vitro development to the blastocyst stage of pronuclear transplanted embryos was observed after fusion in 0.3 M mannitol and PBS, each containing 0.9 mM Ca^{2+}.

CHAPTER I

INTRODUCTION

The production of multiple identical animals is of great interest to scientists conducting experiments in fields ranging from cellular and developmental biology to population genetics. Nuclear transplantation techniques can be used to study, among others, gene regulation, nucleo-cytoplasmic interactions and cell-cycle regulation. Multiple clones could provide the ideal control for experimental conditions thereby reducing the experimental genetic variation to zero (First, 1990). This also allows for the use of fewer animals to achieve statistical validity. These clones would be very useful in environmental, reproductive and nutritional experiments not only because fewer animals would be required, but interactions between genotypes and treatments could be more closely studied.

The livestock industry is also interested in the production of clones to shorten phenotypic selection time and decrease product variation. On a commercial production level, time required to perform progeny tests would be greatly diminished. Selected characteristics such as meat and milk production could be rapidly changed to fit changing industry management and production requirements. The selection of clonal lines descending from a single embryo could be used to produce large numbers of clones possessing specific traits. Production efficiency could be increased since a uniform group of animals would respond similarly to particular feeding and management practices.

Offspring from livestock species have been produced by nuclear transplantation in the sheep (Willadsen, 1986; Smith and Wilmut, 1989), cow (Prather et al., 1987;

Bondioli et al., 1990) and pig (Prather et al., 1989a). Although 8 calves have been derived from a single embryo (First, 1990), the efficiency of cloning via nuclear transfer remains low. Efficiency can be calculated by multiplying the percentage rate of four factors critical in the nuclear transfer process: enucleation x fusion x activation x embryo transfer = % success rate (Stice and Robl, 1988). The success rate of offspring developing from nuclear transplant embryos is approximately 1% in pigs (Prather et al., 1989a), 4% in sheep and cattle (Smith and Wilmut, 1989; Bondioli et al., 1990) and 10% in rabbits (Collas and Robl, 1990).

CHAPTER II

LITERATURE REVIEW

PARTHENOGENESIS

The term parthenogenesis (Gr., "virgin birth") was first used by Richard Owen in 1849 to describe "procreation without the immediate influence of a male". In recent years it has been defined as "the production of an embryo, with or without eventual development into an adult, from a female gamete in the absence of any contribution from a male gamete" (Kaufman, 1979).

Most of the experimental work on mammalian parthenogenesis has been conducted utilizing the mouse as the research model (for reviews see: Beatty, 1957; Graham, 1974; Tarkowski, 1975; Kaufman, 1983). Parthenogenetic embryos can be either haploid or diploid in their chromosome makeup. Four major types of parthenogenones as well as a fifth subtype have been described in artificially activated mammalian eggs (Whittingham, 1980):

1) Haploids with one pronucleus and two polar bodies - oocytes have completed their second maturation division with one haploid set of chromosomes, forming a single pronucleus, while the other set is being extruded as the second polar body.

2) Mosaic haploids with two pronuclei - oocytes accomplish the first maturation division but the second is omitted. This results in cleavage into halves, which give rise to two individual haploid blastomeres. This pathway has been designated as "immediate cleavage" (Braden and Austin, 1954).

3) Heterozygous diploids with one pronucleus - a single pronucleus develops

from the metaphase II chromosomes, followed by DNA replication and subsequent cleavage into two diploid blastomeres.

4) Heterozygous diploids with two pronuclei - oocytes that are activated without emitting the second polar body resulting in the formation of two haploid pronuclei from the two haploid sets of chromosomes. The haploid chromosomes come together on the first cleavage spindle and give rise to a diploid embryo, genetically identical to the heterozygous diploid, with a single pronucleus.

5) Delayed cleavage of heterozygous diploids with two pronuclei - a portion of the oocytes that initially develop two haploid pronuclei undergo a delayed cleavage that takes place between 10 and 24 hours after activation (Graham, 1971; Kaufman and Sachs, 1976). This class of parthenogenones can be identified by the presence of four blastomeres at the time of first cleavage division.

In addition to the major classes mentioned above, other types of parthenogenesis may occur. For example, activating murine oocytes in medium lacking Ca^{2+} and Mg^{2+} ions can result in the second polar body being void of second meiotic division products. Therefore diploid oocytes and immediately cleaved oocytes (each blastomere containing a pronucleus) may be observed containing a normal appearing polar body within the perivitelline space. This occurrence can lead to confusion in assessing parthenogenones, however observance of this event is rare (Kaufman, 1983).

The type of parthenogenetic development depends on the activation method, postovulatory age of ova, osmotic pressure and the temperature of the culture medium. Changes to these conditions may have a substantial effect on the degree of parthenogenesis and on the type of parthenogenetic development (Whittingham, 1980).

Activation procedures usually produce genetically uniform haploids (i.e. one pronucleus and extruded polar body), however, by experimentally blocking second polar body extrusion, parthenogenones containing one diploid or two haploid pronuclei are produced. In addition, by increasing either the dose of activating agent or treatment duration, a greater number of mosaic haploids and both types of diploid parthenogenones result.

Experimental Production of Parthenogenetic Embryos

The first experiments aimed at inducing parthenogenesis in mammals date back to the 1930's in which rabbits were the model system (Pincus and Enzmann, 1937; Pincus, 1939). Parthenogenesis was later confirmed in other species utilizing temperature shock to unfertilized tubal eggs (Chang, 1954; Beatty, 1957) and genetic inactivation of chromosomal sets in the male and female gametes induced by exposure to X-rays or ultraviolet rays (Edwards, 1957a,b). Parthenogenetic development to the blastocyst stage has been reported in rabbits (Pincus and Shapiro, 1940) and mink (Chang, 1957).

More efficient and reliable techniques for inducing parthenogenesis in mammals were devised in the early 1970's. These provided the ability to increase the rate of parthenogenesis which permitted detailed studies of the different developmental patterns that occur in parthenogenones (Tarkowski 1971, 1975; Graham, 1974; Kaufman, 1975a, 1978).

The methods that have been employed for obtaining parthenogenetic embryos can be divided into two groups depending on whether the oocytes are activated directly in situ within the oviducts or removed from the oviducts, cultured and activated in vitro.

Both methods are reliable for the production of parthenogenones of a desired karyotype.

Many different agents are capable of inducing oocyte activation, however, most are toxic to oocytes. It should be noted that activated oocytes containing one normal pronucleus are not always capable of further development. Highly toxic agents as well as harsh treatment can efficiently induce parthenogenesis, however the activated oocyte may not develop beyond the one-cell stage or arrests after 1-3 cleavage divisions. The developmental capacity as well as the incidence of activation must be assessed when attempting to compare the efficiency of different activation techniques.

In Vivo Activation

A variety of techniques have been used to induce murine oocytes to develop parthenogenetically within the oviduct of an anaesthetized mouse. In 1954, Braden and Austin reported inducing parthenogenesis by applying heat shock to the oocytes within the exteriorized oviduct of anaesthetized mice. Cold shock has also been shown to be effective in artificially activating oocytes of various mammalian species, including rat and sheep (Thibault, 1949), hamster (Austin, 1956), ferret (Chang, 1957), and rabbit (Chang, 1952, 1954). Tarkowski et al. (1970) reported an electrical shock applied to the ampullary region of an exteriorized oviduct in an anaesthetized animal could induce parthenogenesis. An advantage of this technique was that postimplantation development to the egg cylinder stage would occur when freshly ovulated oocytes were stimulated by an electric pulse. In addition, the in vivo electrical shock technique was found to be effective in inducing parthenogenesis in hamster eggs with subsequent development to the morula and blastocyst stage (Kaufman et al., 1975a).

Injection of ethanol into an animal was also found to be effective in inducing oocyte activation. An intraperitoneal injection of 25% ethanol 7-9 hours after ovulation induced activation of up to 70% of mouse oocytes (Dyban and Khozhai, 1980). Parthenogenones derived by this method developed to the late morula stage with approximately 12% forming morphologically normal blastocysts. However, development to the egg cylinder stage was not observed and appeared to be the stage at which degeneration occurred.

Finally, data indicate anaesthetic agents can induce oocyte activation in vivo. Chloroform, nitrous oxide and nembutal have been used to parthenogenetically activate rat oocytes while still in the oviduct (Austin and Braden, 1954). Ether (Braden and Austin, 1954) and avertin (Kaufman, 1975b) have also been used to activate mouse oocytes in vivo.

In Vitro Activation

Many techniques have been developed to induce artificial activation of mammalian oocytes in vitro. As in the in vivo studies, the majority of the in vitro data has been derived from research using the mouse as the mammalian model. A number of mechanical and environmental factors are involved in the in vitro induction of parthenogenesis. The include mechanical disruption or disturbance of the vitelline membrane, altering pH and temperature as well as altering the chemical composition of the culture medium. In addition, age of oocyte and the species or strain used are important factors that influence parthenogenetic frequency.

Mechanical Activation

In 1977, Uehara and Yanagimachi reported that over 80% of hamster oocytes were artificially activated after a glass microneedle had penetrated the vitelline membrane. This disruption of the oolemma caused cortical granule exocytosis, extrusion of the second polar body and formation of a single pronucleus.

Penetration and injection of PBS + polyvinylpyrrolidone (sham treatment) also has been shown to activate hamster oocytes (Sinosich et al., 1990). Stice and Robl (1990) demonstrated that a factor contained in homogenized rabbit sperm fractions was able to activate 73% of rabbit oocytes. In addition, the sham injection (control) of PBS + 0.86 mM $CaCl_2$ + 4 mg/ml bovine serum albumin caused activation in 12% of the rabbit eggs (Stice and Robl, 1990).

Environmental Activation

In vitro environmental factors play an important role in the parthenogenicity of mammalian oocytes. Johnson et al., (1990) reported that brief exposure to acidified Tyrodes medium (pH=2.5) induces activation of mouse and human oocytes. Subjecting mouse oocytes to cold shock treatment by reducing the temperature to 1°C for 4.5 min has also been reported to activate mouse oocytes (Shaw and Trounson, 1989).

Chemical Activation

Enzymes have also been used to activate mouse eggs. A 5 min exposure to culture medium containing hyaluronidase has been shown to be sufficient to activate mouse eggs 16 -25 h after human chorionic gonadotropin (HCG) injection (Graham, 1970; Kaufman, 1975a). Incubation of mouse oocytes in a 0.25% solution of pronase

for 10-15 min has also been reported to result in activation (Kaufman, 1975a; Johnson et al., 1990).

Altering the concentation of certain divalent cations can induce oocyte activation. Cytosolic injection of Sr^{2+} or Ba^{2+}, as well as incubation in media containing these cations, has been shown to mimic Ca^{2+} dependent events associated with oocyte activation (Fulton and Whittingham, 1978; Marcus, 1990). Culture medium devoid of Ca^{2+} and Mg^{2+} ions has been reported to cause parthenogenic activation in mouse (Surani and Kaufman, 1977), rat (Miyamoto and Ishibashi, 1975), and hamster eggs (Whittingham and Siracusa, 1978). It has been proposed that Ca^{2+}-free medium induces alteration of the physical properties of the oolemma thereby inducing Ca^{2+} release from internal stores (Whittingham, 1980). Further support of this observation was provided by Whittingham (1980) who reported that excessive levels of Ca^{2+} in the culture medium inhibited activation of mouse eggs.

The Ca^{2+}, Mg^{2+} and H^{+} ionophore A23187 activates oocytes by causing an intracellular release of Ca^{2+}. Approximately 90% of hamster eggs were found to have been activated after exposure to 3 μM concentration of A23187 for 2 min (Steinhardt et al., 1974). Ware et al., (1989) reported activation rates of 96% and 100% in bovine oocytes subjected to 1 μM and 10 μM A23187, respectively. Although activation rates have been high utilizing this method, it is not without possible detrimental effects. It was reported in the original work with hamster eggs (Steinhardt et al., 1974) that A23187 delayed mitosis, caused abnormal cleavage and that protein synthesis in the treated eggs was not maintained.

Alcohol Activation

Perhaps the most widely used method of inducing parthenogenesis is by ethyl alcohol. The procedure reported for in vivo activation has been modified for in vitro activation of mouse (Kaufman, 1982; Cuthbertson, 1983) and bovine oocytes (Nagai, 1987). In many strains of mice, the activation frequency of oocytes exposed to 7% absolute ethanol in PBS for 7 min is relatively high (80-100%). In addition, a high percentage of the eggs activated by this method develop to the blastocyst stage (Kaufman, 1983). Bovine oocytes removed from ovarian follicles (1-5 mm), matured in vitro for 27 h and exposed to 7% ethanol for 7 min were induced to activate and form pronuclei at a rate of 60% compared to 3% in non-treated controls (Nagai, 1987).

Electrical Activation

Electrical stimulus has been reported to activate mouse (Tarkowski et al., 1970) and hamster oocytes (Kaufman et al., 1975a) in situ. Electric pulses have been applied to oocytes in vitro to study artificial activation in the mouse (Onodera and Tsunoda, 1989; Didion et al., 1990; Marcus, 1990), rabbit (Stice and Robl, 1988; Onodera and Tsunoda, 1989; Didion et al., 1990; Ozil, 1990), pig (Didion et al., 1990), sheep (Smith and Wilmut, 1989), and cow (Kono et al., 1989; Ware et al., 1989).

Onodera and Tsunoda (1989) reported that a 1.5 $kV \cdot cm^{-1}$ DC pulse applied for a duration of 100 μsec activated 78% of CD-1 strain mouse oocytes. Stice and Robl (1989) reported a 52% rate of activation when rabbit oocytes were exposed to a 1.6 $kV \cdot cm^{-1}$ 60 μsec pulse.

Electric pulses with field strengths of 1 to 2.5 $kV \cdot cm^{-1}$ have also been used to induce fusion between two adjacent cell membranes in vitro (Zimmermann and

Vienken, 1982; Kubiak and Tarkowski, 1985). Electrofusion has become important in cloning of early mammalian embryos by providing an efficient means of introducing a foreign nucleus into an enucleated oocyte as well as activating the recipient oocyte's cytoplasm.

Success of cloning via nuclear transplantation is dependent upon the activation of the recipient oocyte, which is essential for reprogramming the donor nucleus and its subsequent development (Robl and Stice, 1989; Prather and First, 1990b). Therefore, experiments are currently being conducted to maximize the cell fusion efficiency in conjunction with the ability to activate the egg, thereby initiating final meiotic events.

Role of Ca^{2+} at Fertilization

Upon sperm-egg fusion a dramatic change of events takes place within the fertilized egg. Striking changes in membrane potential and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ have been observed in various invertebrate and vertebrate species (Cuthbertson et al., 1981; Epel, 1982; Jaffe, 1983). This $[Ca^{2+}]_i$ oscillation triggers cortical granule exocytosis and also initiates events leading to egg activation, defined as the resumption of second meiosis and second polar body formation. In the sea urchin, the change in membrane potential is important for a fast electrical block to polyspermy while the increase in $[Ca^{2+}]_i$ causes cortical granule exocytosis and subsequent development of the fertilization envelope (permanent block to polyspermy). Transient $[Ca^{2+}]_i$ increase is implicated in causing NAD kinase activation and an increase in O_2 consumption (Epel, 1978). In mammalian eggs studied to date, there is no electrical block to polyspermy (Miyazaki and Igusa, 1982; Jaffe, et al., 1983; McCulloh et al., 1983). The changes in membrane potential include recurring transient

hyperpolarization responses (Miyazaki and Igusa, 1981; Igusa et al., 1983; McCulloh et al., 1983). This hyperpolarization response to fertilization is due to transient increases in $[Ca^{2+}]_i$ which causes increase in K^+ conductance (Igusa and Miyazaki, 1986).

Periodic $[Ca^{2+}]_i$ oscillations occur for at least 4 h following fertilization in the mouse (Cuthbertson and Cobbold, 1985) and at least 30 min in the hamster (Miyazaki et al., 1986). Periodic transient waves arise due to release of Ca^{2+} from intracellular stores, most probably from the smooth endoplasmic reticulum (Cuthbertson and Cobbold, 1985; Igusa and Miyazaki, 1986).

Although the mechanism by which sperm-egg fusion induces a release of intracellular Ca^{2+} remains unclear, activation of guanosine triphosphate (GTP-binding protein = G protein), inositol 1,4,5-triphosphate (IP3), as well as Ca^{2+} mediated Ca^{2+} release (CICR) are thought to be responsible for causing periodic $[Ca^{2+}]_i$ waves (Miyazaki, 1988a; Swann et al., 1989).

Miyazaki (1988b) proposed that sperm-egg binding activates a G protein that is involved in the subsequent release of Ca^{2+} from intracellular stores. G proteins have been reported to activate phosphodiesterase which in turn mediates cleavage of phosphatidylinositol 4,5-biphosphate (PIP2) into IP3 and diacylglycerol (reviewed by Stryer and Bourne, 1986). IP3 is an intracellular second messenger that acts on the endoplasmic reticulum to release stored Ca^{2+}.

G Proteins

G proteins can be activated through microinjection of guanosine-5-0-3-thiotriphosphate (GTP gamma S), a GTP analog. Miyazaki (1988b) microinjected GTP gamma S into hamster oocytes at an ooplasmic concentration of 12 μM. A subsequent

$[Ca^{2+}]_i$ wave was recorded in each of the oocytes lasting from 160 to 200 sec. Periodic $[Ca^{2+}]_i$ waves were produced when the intracellular concentration of GTP gamma S was more than 50 μM.

Injection of guanosine-5'-0-(2-thiodiphosphate) GDP beta S, (a GTP antagonist) into hamster oocytes inhibited both sperm induced and GTP gama S induced $[Ca^{2+}]_i$ transients. These data infer that alterations in intracellular $[Ca^{2+}]_i$ at fertilization involves a G protein mediated process (Miyazaki, 1988b).

Inositoltriphosphate

The intracellular phosphoinositide second messenger system has been implicated in governing reinitiation of DNA replication and playing an important role in oocyte activation (Whitaker, 1989). IP3 is a component of this messenger system and acts on the endoplasmic reticulum to cause a release of stored Ca^{2+}. The rise in $[Ca^{2+}]_i$ is thought to initiate biological signal transduction processes which control such cell functions as proliferation, metabolism and secretion (Reviewed by Epel, 1978; Berridge and Irvine, 1984; Jaffe, 1985). IP3 has been shown to induce an intracellular Ca^{2+} release in a variety of cell types (Berridge and Irvine, 1984).

Research conducted on oocytes has shown that IP3 induces intracellular Ca^{2+} release in the amphibian (Busa et al., 1985; Han and Nuccitelli, 1990), sea urchin (Whitaker and Irvine, 1984; Turner et al., 1986; Steinhardt and Alderton, 1988) and hamster (Miyazaki, 1988b).

Miyazaki (1988b) reported that a single injection (2 nM cytoplasmic volume) of IP3 into the hamster ooplasm induces an immediate $[Ca^{2+}]_i$ rise with a duration of 13-18 sec. A single injection of IP3 usually produced only one $[Ca^{2+}]_i$ rise while

continuous leakage of IP3 from the injection pipette produced a series of $[Ca^{2+}]_i$ transients (Miyazaki et al., 1988).

ARTIFICIALLY INDUCED CELL FUSION

The ability to fuse two cell membranes together to form a single membrane-bound unit has allowed significant advances in membrane research, membrane reconstitution, and genetic mapping. Both somatic cell hybridization and genetic engineering provide a means of modifying plant and animal cells in order to study cell mechanisms of development and synthesis of gene products. For example, if lymphocytes are fused with myeloma cells, hybridoma cells are produced which are capable of synthesizing monoclonal antibodies with a determined antigenic specificity (Bellve´ and Moss, 1983). Antibody producing hybridoma cells are routinely used to produce monoclonal antibodies for clinical diagnostics as well as a wide range of biological and medical research interests. In vitro cell-to-cell fusion can be used for studying membrane interaction during various cellular processes. These include studies related to exo- and endocytosis, sperm-egg fusion at fertilization and production of gamete hybrids.

Fusion of mammalian cell membranes is achieved through several methods, including: a) incubation with uranyl ions or other rare earth metals (Majumdar et al., 1980); treatment with calcium and phosphate ions (Baker and Kalra, 1979); c) utilizing viral fusogens (Knutton, 1978; White et al., 1983); d) incubation with polyethylene glycol (Knutton, 1979); and e) by subjecting cells to an electric field procedure termed "electrofusion" (Zimmermann and Vienken, 1982). Viral fusogens,

polyethylene glycol, and electrofusion are the most widely used methods of inducing cell membrane fusion as discussed below.

Polyethylene Glycol

Polyethylene glycol (PEG) is a potent fusogenic compound that has gained wide acceptance in the production of cell hybrids (Wojcieszyn et al., 1983). PEG has the capability of fusing a wide variety of cell membranes, including fusing together unrelated cell types to form interspecific and interkingdom hybrids (Ward, 1984). The advantages of using PEG over other fusogens are its reproducibility, inexpensiveness, availability, and ease of handling. However, the major drawback of PEG is that different cells have varied susceptibility to the fusogenic effects of PEG (Wang et al., 1982). In a given cell line, efficient cell fusion is obtained through a narrow range of PEG concentrations, with little fusion occurring below a threshold level, and toxicity occurring in more concentrated solutions (Roos and Davidson, 1980).

PEG has been used in the fusion of murine oocytes (Gulyas et al., 1984), murine two-cell embryos (Spindle, 1981), mouse, hamster, and rabbit sperm to both mouse and hamster oocytes (Didion and Graves, 1988).

PEG induced fusion protocols generally utilize a short (1-3 min) incubation period in a 25-50% w/v of (1000-6000 MW) PEG. After the co-incubation period, PEG is removed through washing by centrifugation. The centrifugation step allows removal of the PEG containing supernatant and aids in the fusion process by increasing the cell-cell contact. Cells are then incubated in PEG-free medium to allow sealing of membrane bilayer. The PEG induced fusion occurs only after its removal from the medium (Hui et al., 1985). Membrane fusion is thought to occur due to the ability of

PEG to bind and structure H_2O molecules. PEG dehydrates membranes by competing for free H_2O molecules. This competition causes a change in the dielectric property of H_2O, which in turn, causes defects in the phospholipid bilayer. PEG differs from other dehydrating agents by its abilities to bind phospholipids and accumulate membrane proteins. Furthermore, it is possible that PEG alters the ion exchange rate between bound and free H_2O molecules (Boni & Hui, 1987).

The mode of action by which PEG induces fusion has been postulated based on cellular and artificial lipid membrane systems. Membrane proteins are forced into clusters, leaving bare patches of high lipid content (Maroudas, 1975). This defect of the membrane bilayer is caused by local dehydration and changing of the dielectric constant of water. In addition, the decrease in polarity of the medium facilitates closer contact and therefore allows subsequent fusion of the phospholipid bilayers. A concentration of PEG that is excessively high would deplete the cells of their water content and lead to leakage of cellular components and subsequent decrease in cell viability (Boni & Hui, 1987).

Virus Induced Cell Fusion

Virus induced cell fusion has been reported to occur under pathological conditions for more than 100 years. However, cell fusion by various viruses was not accomplished in tissue culture systems until the 1950's (Okada et al., 1957; review, Papahadjopoulos et al., 1979). Among the many viruses tested, Sendai virus is the most widely used (Poste and Pasternak, 1978). This is due to the relative ease of preparation, moderate stability, is easily inactivated with ß-propiolactone, and is

considered nonpathogenic to humans. Viral fusion is accomplished with inactivated virus particles which do not have the capability to replicate.

Sendai virus is a myxovirus of Group II and has the synonyms of Parainfluenza I and HVJ (hemagglutinating virus of Japan). For use in cell fusion experiments, Sendai virus is normally grown to a high titer [measured in hemagglutinating activity units (HAU)] in the allantoic cavity of embryonated chicken eggs (Giles and Ruddle, 1973). The number of HAU's required for each experiment is subject to the fusion activity of the virus preparation and the ability of the cells to fuse together. Generally, 1,000-2,500 HAU of virus are used to induce membrane fusion (Papahadjopoulos et al., 1979).

The precise mechanism used in virus induced cell fusion is unknown. It appears that fusion can be mediated by viral proteins incorporated within the plasma membrane or by the virion itself. Inactivated Sendai virus induces membrane fusion through the action of the virion itself. Cell membranes must be exposed to large number of virions in order to initiate cell-cell fusion. The virions are thought to cause membrane lesions which increase permeability and subsequently cause the formation of cytoplasmic bridges. These bridges enlarge, possibly due to the fluid nature of the membrane bilayer, and mixing of the cytoplasm occurs (Spear, 1987).

Sendai virus has been used successfully to fuse embryonic cells in nuclear and cytoplasmic transplantation experiments (McGrath and Solter, 1986). Karyoplasts (nuclei with a small amount of cytoplasm surrounded by cell membrane) containing four and eight-cell mouse blastomere nuclei were fused with enucleated two-cell

blastomeres by 2,500 HAU of Sendai virus. Live offspring from both the four and eight-cell nuclei were produced (Kono and Tsunoda, 1989).

Electric Field Induced Cell-to-Cell Fusion

The electric field induced fusion technique provides an interesting alternative to the previously mentioned fusion methods. The electro fusion technique is based on two distinct phases:

(a) Two cells are brought into close membrane contact by the application of a nonuniform, alternating electric current (AC) field of low intensity.

(b) Subsequent fusion is initiated by a high intensity field pulse ($kV \cdot cm^{-1}$) of short duration (usually in the μsec range). The direct current (DC) pulse results in a temporary structural change in membrane surface that is perpendicular to the electrodes.

Electric field induced cell fusion requires very close membrane contact between cells. Various factors affiliated with the hydrophilic membrane constituents, such as water molecules, brownian movement, and the net negative charge, generally prevent cells from comming into close membrane contact (Miles and Hochmuth, 1987). These repellant forces are temporarily overcome by the AC field. A nonuniform AC field causes the cells to form dipoles, which allows the opposite charged ends to come into close contact.

The fusion process is initiated by a single or several DC field pulses varying from 3 to 200 μsec in duration (depending on species and intensity). The field strength required for reversible membrane breakdown is in the order of 0.5 to 10 $kV \cdot cm^{-1}$ (Zimmermann et al., 1976).

Electric-induced breakdown causes conductive and permeable pores (6 nm in diameter) to form within the membrane (Zimmermann and Vienken, 1982). These pores are able to persist from micro-seconds to minutes depending on temperature, location of membrane breakdown, and pulse strength and duration. The formation of pores causes a considerable increase in membrane permeability. This increased permeability allows intracellular and extracellular exchange of cell components. Pores formed in membranes that are in close contact, join together and reform the lipid bilayer. These intercellular channels allow mixing of cytoplasm and result in the fusion of the two cells (for review see: Zimmermann and Vienken, 1982).

Cell fusion experiments generally use low-ionic-strength medium in order to decrease the amount of current flowing through the cells. Common electrofusion medium used include 0.3 M mannitol (Ozil and Modlinski, 1986; Stice and Robl, 1988), 0.3 M mannitol + 0.1 mM MgSO4 + 0.05 mM $CaCl_2$ (Willadsen, 1986; Smith and Wilmut, 1989) or a phosphate-buffered sucrose solution (Miles and Hochmuth, 1987). Since these sugars move across the post-electrofusion membrane much more slowly than sodium, they are substituted for sodium chloride to maintain the osmotic pressure while decreasing the conductive salt concentration (Zimmermann and Vienken, 1982; Miles and Hochmuth, 1987).

Kubiak and Tarkowski (1985) demonstrated the efficiency of electrofusion on 2-cell mouse blastomeres. Two pulses of 1 $kV \cdot cm^{-1}$ for a duration of 250 µsec resulted in a fusion rate of 88% (66/75) without visible damage to the 2-cell embryos.

NUCLEAR TRANSPLANTATION: CLONING OF MAMMALIAN EMBRYOS

Blastomere Isolation & Embryo Splitting

There are three methods currently used to produce genetically identical animal clones. Blastomere isolation, embryo splitting and nuclear transplantation. The first entails isolating individual blastomeres from early pre-compaction stage embryos. Blastomeres isolated from 2-cell or 4-cell embryos have the ability to develop into a complete individual (Willadsen et al., 1981). Development of isolated blastomeres from more advanced stages of development rarely develop beyond early embryonic stages (Moore et al., 1968; Rossant, 1976). The second method involves dividing a morula or blastocyst stage embryo into two to four segments (Willadsen et al., 1981; Ozil et al., 1982; Lambeth et al., 1983; Voelkel et al., 1985). The splitting of late preimplantation embryos is technically simple and is being used commercially to produce twin clones in cattle (Baker & Shea, 1985). The number of clones that can be produced through these two methods is limited due to the fact that the developmental cell cycle schedule remains the same, while the number of embryonic cells per embryo half or quarter segment has been reduced (Tarkowski, 1975; Rossant, 1976). For example, a normal rabbit embryo completes approximately seven cell cycles prior to reaching the blastocyst stage. However, an isolated 8-cell blastomere will complete only four cell cycles prior to forming a blastocyst and contain only sixteen cells. These blastocysts do not have enough cells to allocate to the differentiating tissue types and therefore are usually reabsorbed (Stice & Robl, 1989). When embryos are divided into more than quarters, the number of cells required for differentiation into inner cell mass and trophectoderm cells is insufficient resulting in embryonic death and reabsorption (Tarkowski and

Wroblewska, 1967; Willadsen, 1982). Therefore, producing clones by means of blastomere isolation or splitting late preimplantation embryos is limited to the production of two to four animals.

Nuclear Transplantation

Transplantation of a nucleus from an embryonic cell into an oocyte in which the maternal metaphase II chromosomes have been removed is a method by which multiple offspring can be produced. This was first accomplished in amphibians by transplanting blastula and gastrula cell nuclei into enucleated eggs to produce cloned offspring in frogs (Briggs and King, 1952; Gurdon, 1964). This work provided early impetus to develop methods for cloning mammals. Modlinski (1978) developed a procedure for microsurgically inserting isolated mouse nuclei into murine embryos.

Illmensee and Hoppe (1981) modified this technique and reported that live murine offspring were produced after transfer of inner cell mass (ICM) nuclei into enucleated fertilized zygotes. They developed a method in which the cell membrane of ICM and trophectoderm cells was ruptured to allow aspiration of the nucleus into a micropipet. The nucleus was then injected directly into the cytoplasm by piercing the plasma membrane of a recipient fertilized zygote with the micropipet. Once the nucleus was injected, the pipet was used to remove the recipient zygote's pronuclei from the cytoplasm. This procedure proved to be technically difficult and resulted in only 39% of the embryos surviving micromanipulation. In addition, attempts to reproduce their results of live offspring produced by direct injection of ICM nuclei into the cytosol have failed (McGrath and Solter, 1983; Surani et al., 1984)

McGrath and Solter (1983) published a procedure which increased survivability

post micromanipulation. The technique involved the removal of nuclei or chromosomal material from a cell without rupturing the cell membrane. In this method, a micropipet was placed adjacent to the nucleus without penetrating the plasma membrane. Gentle aspiration allowed the removal of the nucleus which remained in a membrane encased karyoplast. The membrane bound karyoplast was then inserted through the zona pellucida of an unrelated zygote and ejected from the micropipet thereby leaving an enulceated cytoplast and adjacent karyoplast inside the zona pellucida. The karyoplast and cytoplast were then fused together via the fusogenic activity of Sendai virus. Because the plasma membrane was not penetrated, the survival rate remained high.

Successful formation of karyoplasts and cytoplasts requires that the cell membranes to be resilient to manipulation procedures. This is facilitated through the use of cytoskeletal inhibitors, cytochalasin B and colchicine, in the manipulation medium.

Cytochalasin inhibits the function of microfilaments by binding to one end of the actin filament and preventing polymerization. This disruption of microfilaments causes the membrane to become less rigid, thereby allowing nuclei or cytoplasm to be removed from cells with minimal damage to membrane. The negative effect of cytochalasin on mammalian cells include decreased polysaccharide synthesis and inhibition of sugar transport (Granholm and Brenner, 1976).

Colchicine inhibits microtubule polymerization by displacing guanosine triphosphate. Lack of guanosine triphosphate inhibits formation of the meiotic and mitotic spindle apparatus. The addition of colchicine to manipulation medium is necessary when a large number of microtubules are present in the cytoplasm, such as

pronuclear stage embryos. However, it is not required in enucleating a metaphase II oocytes since the majority of the microtubules are located in the meiotic spindle (Schatten et al., 1985).

The nuclear transplantation procedure developed by McGrath and Solter (1983) has been used in experiments studying dedifferentiation of nuclear transplant embryos as well as development of androgenetic and gynogenetic embryos (Barton et al., 1985; Surani et al., 1986). It has also been adapted for the production of sheep (Willadsen, 1986; Smith and Wilmut, 1989), cow (Robl et al., 1987; Prather et al., 1987; Bondioli et al., 1990), pig (Prather, 1989a) and rabbit (Stice and Robl, 1988; Collas and Robl, 1990) offspring with one modification. Electrofusion has replaced the fusogenic Sendai virus as method of choice for fusing karyoplasts and cytoplasts in nuclear transplantation (Willadsen, 1986).

NUCLEAR REPROGRAMMING

The transfer of pronuclei between two pronuclear stage embryos requires little or no reprogramming of the nuclear material. The highest developmental rates are achieved when pronuclear and 2-cell stage nuclei transferred into cytoplasts of the same cell-division stage (Robl et al., 1986,1987; Prather et al., 1989a; Smith et al., 1990). Since the cell cycle program is essentially the same and the cytoplasmic constituents are similar, the transferred nuclei do not require their cell cycle to be reprogrammed. This is not the case, however, of later stage embryonic nuclei. As the cell cycles become asynchronous the developmental potential is greatly diminished. In order to clone embryos from more advanced stages, the nucleus must be reprogrammed to act as if it were a recently fertilized zygote.

The cytoplasmic factors necessary to reprogram transplanted nuclei appear to be restricted to the metaphase II oocyte. Transfer of murine (McGrath and Solter, 1983) and bovine (Robl et al., 1986) 2-, 4-, and 8-cell blastomeres to enucleated pronuclear stage cytoplasm resulted in limited development in vitro and failed to produce offspring. When murine 8-cell blastomeres were transferred to the cytoplasm of enucleated 2-cell blastomeres, 51% (Robl et al., 1986) and 35% (Tsunoda et al., 1987b) developed to the blastocyst stage in vitro and 4 offspring were produced (Tsunoda et al., 1987b). In vitro development of 8-cell nuclei transplanted into 2-cell cytoplasts resulted in significantly fewer numbers of cells at the blastocyst stage, as well as premature blastocoel formation. These data indicated that the inherent cell cycle program of the transferred nuclei was not altered.

A critical factor in the success of cloning via nuclear transplantation is being able to reprogram a transplanted nucleus to function like a 1-cell zygote. This appears to occur only when nuclei are transplanted into the cytoplasm of enucleated activated oocytes (ooplasts). The mechanisms involved in reprogramming transplanted nuclei are not completely understood, however morphological and nuclear protein modifications have been observed (reviewed by Prather and First, 1990a).

Age of Recipient Oocyte

The age of the oocyte used as a recipient for transplanted nuclei has a significant impact on successful nuclear reprogramming and subsequent embryonic development. In order for successful development to be initiated, the recipient ooplasm has to undergo release from meiotic arrest (become activated). Postovulatory age directly effects normal physiological functions of the oocyte. Postovulatory aging in murine

oocytes induces breakdown of the second meiotic spindle, which inhibits chromosomes from organizing in a metaphase plate (Webb et al., 1986). In vivo matured murine oocytes exhibit a stable metaphase II distribution of cortical granules 13-18 h after HCG. Major alterations in the distribution of cortical granules was observed in 28% and 83% of oocytes recovered 24 h and 32 h after HCG injection, respectively (Ducibella et al., 1990). Normal structure and physiological competence are maintained for only a brief period of time after maturation. Bovine oocytes cultured in vitro for 30 h exhibited a significant rate of spontaneous activation (21%), indicating changes in cytoplasmic factors required to maintain the oocytes in meiotic arrest (Ware et al., 1989). It has long been suggested that an increase in polyspermy associated with aged oocytes is related to deteriorating structural and cytoplasmic factors (Austin, 1970; Szollosi, 1975).

In nuclear transfer experiments, activation of the recipient ooplast is accomplished by applying an electrofusion pulse, which induces fusion of the karyoplast to the ooplast as well as activates the ooplasm. One important factor in nuclear transplantation experiments is being able to activate an ooplast which is still developmentally competent. Data from murine nuclear transfer experiments indicate that recently ovulated oocytes activate at a lower rate when oocytes were recovered 15 h (3%) vs 25 h (42%) after HCG injection (Robl and Stice, 1989). In rabbit nuclear transplantation experiments, oocytes manipulated 18.5 h after HCG exhibited a higher enucleation rate and fused at a higher rate than oocytes manipulated 24 h after HCG (60% vs 3% and 55% vs 26%, respectively; Collas and Robl, 1990). However, rate of activation in the 18.5 h group was significantly lower than those in the 24 h group (3% vs 37%,

respectively). Although maximum activation rates are obtained in aged oocytes, development of the transplanted nuclei in aged oocytes is decreased. Bovine oocytes manipulated 48 h after first detection of estrus are less able to support development after nuclear transplantation than oocytes collected 36 h after estrus detection (Prather et al., 1987).

Nuclear Swelling

It is current theory that cytoplasmic components from the recipient cytoplasm pass through the nuclear membrane and initiate morphological changes. One morphological event is characterized by a swelling of the nucleus (Gurdon, 1964; Hoffner and DeBeradino, 1980; Czolowska et al., 1984). This is probably due to cytoplasmic proteins entering the nucleus which have been reported to modify the chromatin (Meriam, 1969; Gurdon, 1986).

Czolowska et al., (1984) transferred mouse thymocyte nuclei into activated and nonactivated metaphase II oocytes. Thymocyte nuclei transferred to nonactivated oocytes underwent premature chromosome condensation with individualization of chromosomes. In contrast, thymocyte nuclei transferred to activated oocytes underwent decondensation and nuclear swelling similar to pronuclear formation. Nuclear volume increased up to 200 times during 24 hours in culture. It was also noted that there seemed to be a 1.5 h time period at the time of activation (30 min prior, 60 min post) in which the nucleus would respond by swelling.

Stice and Robl (1988) monitored nuclear swelling in rabbit nuclear transplant experiments in which 8-cell nuclei were transferred to mature enucleated oocyte cytoplasts and enucleated pronuclear cytoplasts. Nuclear diameter increased from 9.6

± 0.7 μm to 22 ± 1.0 μm within 6 hours after transfer to oocyte cytoplasts. Transfers of 8-cell nuclei to pronuclear cytoplasts failed to undergo nuclear swelling, indicating an inability to reprogram the nucleus.

Prather et al., (1990) measured nuclear swelling and cytoplasmic volume and its effect on transplanted pig nuclei. Blastomeres from 4-, 8-, and 16-cell embryos were fused to activated oocyte cytoplasts. The diameter of the 4-, 8-, and 16-cell nuclei swelled from 18.3, 14.3 and 13.0 μm to 26.9, 27.3 and 27.2 μm, respectively. The degree of nuclear swelling does not appear to be related to cytoplasmic volume and it was concluded that sufficient ooplasmic components mediating nuclear swelling remained when up to half of the ooplasm was removed during the enucleation procedure.

Nuclear Lamins

Nuclear lamins are one class of proteins found to migrate from the cytoplasm into the nucleus when the nuclear membrane reassociates after meiosis or mitosis (Gerace and Blobel, 1980). It is postulated that these lamins provide an architectural framework for the nuclear membrane (Gerace et al., 1978) and mediate the attachment of interphase chromatin domains at the nuclear periphery (Hancock and Hughes, 1982; Lebkowski and Laemmli, 1982). Replication, transcription and control of gene activity have been suggested to depend upon the anchorage of DNA loops to the nuclear membrane structure (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986). This lamin framework may have an important effect on chromatin organization during interphase and has been implicated in the regulation of nuclear membrane structure during the cell cycle (Gerace, 1986).

Three major lamins, A, B, and C (M_r 70, 67 and 60 kDa, respectively) are found to reside on the inner nuclear membrane (Gerace et al., 1978). The A/C type of nuclear lamins becomes undetectable after the transition from maternal to zygotic control of development in the mouse (Schatten et al., 1985). During the second cell cycle, synthesis of lamin A ends and synthesis of lamin B begins (Houliston et al., 1988). However, lamin A is synthesized once again in the postimplantation day 8 embryo (Stewart and Burke, 1987).

A similar temporary loss of the A/C epitope is found to occur at the maternal to zygotic transition in the pig and cow (Prather et al., 1989b). This transition to zygotic control occurs after the 2nd cell division (4-cell stage) in the pig and 3rd cell division (8-cell stage) in the cow (Reviewed by Telford et al., 1990).

Prather et al., (1989b) transferred 16-cell stage porcine embryonic nuclei into enucleated metaphase II oocytes and found that the nucleus under zygotic control was able to acquire the A/C epitope once again. It was hypothesized that the transferred nucleus was able to take up the A/C lamin proteins from the ooplasm and that this change in nuclear lamin composition may have a role in nuclear reprogramming.

DNA Structural Rearrangement

In addition to nuclear swelling and exchange of cytoplasmic proteins, regulation of DNA synthesis occurs. Nuclei in the G_1 or S, but not G_2 (DeRoeper et al., 1977) phase of the cell cycle initiate or continue DNA synthesis post transfer to a metaphase II oocyte (Naish et al., 1987). In pronuclear stage mouse embryos, DNA synthesis and the start of S phase begins 5 to 6 hours after pronuclear formation (Luthardt and Donahue, 1973; Kirshna and Generoso, 1977) and lasts approximately 4 h. In addition,

the male pronucleus begins and ends DNA synthesis earlier than the female pronucleus (Luthardt and Donahue, 1973).

DNA replication at the pronuclear stage allows cleavage while maintaining the diploid state of the embryo. As nuclei become more developmentally advanced, the length of their cell cycle increases. As these advanced nuclei are transferred to recipient cytoplasts, abnormal DNA replication becomes more likely. Therefore, nuclei from more developmentally advanced cell cycle stages are more likely to result in chromosomal abnormalities after transfer (Gurdon, 1986).

Cell Cycle

The stages of the cell cycle in somatic cells are characterized by the state of the nucleus. The proliferating cell nucleus can be in one of four phases: G_1, S, G_2 or M. During S phase, the nucleus is synthesizing DNA. During M phase the nucleus dissolves and the chromosomes condense as they undergo mitosis. The G_1 phase is the gap between M and S, and G_2, the gap between S and M. When somatic cells cease to divide, they stop at a specific stage in the cell cycle, usually at the beginning of G_1. Immature mammalian oocytes are arrested in a state analogous to G_2. The first sign of maturation is dissolution of the nuclear membrane (germinal vesicle breakdown) which occurs immediately after S phase. Maturing oocytes then undergo meiosis and extrude the first polar body. The oocytes are then arrested in M phase of the second meiotic division prior to fertilization.

Chromosomal abnormalities resulting from incomplete DNA replication may occur when nuclei currently in the S Phase are transferred to ooplasts. It has been proposed that once DNA synthesis begins, the nuclear envelope must remain intact

(Blow and Laskey, 1988). If alterations in the nuclear membrane result, a licensing factor enters the nucleus and initiates synthesis of DNA which has only recently undergone replication.

Electrofusion procedures used in nuclear transplantation experiments induce short term disruption and pore formation in the plasma membrane. Disruption may also occur in the nuclear membrane, thereby allowing licensing factors to enter the nucleus and cause replication of previously replicated DNA (Blow and Laskey, 1988). Such a nucleus could then become polyploid, resulting in limited embryonic development. It may be important that the transferred nucleus has not entered S or G_2 phase, and therefore DNA replication in order to maximize development.

Nuclear transfer experiments in mammals have shown an abnormal rate of embryonic loss during early pregnancy. In bovine nuclear transfer, donor nuclei from 2-cell to 16-cell stage embryos resulted in 20% of the fused embryos developing to morula / blastocysts (Prather et al., 1987). Only 7/19 of the morula/blastocysts resulted in pregnancies as confirmed by ultrasound between day 21 and 30. Of the confirmed pregnancies, 5/7 aborted and returned to estrus by day 88. Bondioli and coworkers (1990) reported a similar trend in early embryonic mortality when 32-cell blastomeres were used as nuclei donors. Prather et al., (1989a) used 4-cell porcine blastomeres as donor nuclei and reported that of 3 gilts receiving 10 or more nuclear transfer embryos, all 3 had extended estrus cycles (28, 52, and 72 days). It is possible that embryonic mortality may be a result of chromosomal abnormalities incurred because of nuclear transplantation.

CHAPTER III

ELECTROFUSION INDUCED CHANGE IN INTRACELLULAR Ca^{2+} AND ITS EFFECT ON MURINE OOCYTE ACTIVATION

INTRODUCTION

It has been established in various species that a transient increase in $[Ca^{2+}]_i$ is associated with fertilization (Reviewed by Miyazaki, 1988a; Epel, 1990). An increase in $[Ca^{2+}]_i$ results in cortical granule exocytosis and initiates resumption of second meiosis with subsequent second polar body extrusion (Reviewed by Jaffe, 1985). Artificial activation of oocytes is affected by both internal and external $[Ca^{2+}]$. By altering the $[Ca^{2+}]$ inside the cell as well as without, parthenogenesis can be induced. Intracellular Ca^{2+} levels have been altered by inducing ionic stress upon oocytes by culturing in Ca^{2+} and Mg^{2+} free media. Incubation in Ca^{2+}, Mg^{2+} free media induces resumption of meiosis in oocytes of the mouse (Surani and Kaufman, 1977), rat (Miyamoto and Ishibashi, 1975) and hamster (Whittingham and Siracusa, 1978). Ionic stress due to excessive levels of Ca^{2+} in culture media has also been implicated in altering $[Ca^{2+}]_i$, since it induces parthenogenesis in mouse oocytes (Whittingham, 1980). In addition, a high proportion of mouse oocytes have been shown to initiate resumption of meiosis when Ca^{2+} is removed from culture media and replaced with equal concentrations of Ba^{2+} or Sr^{2+} (Fraser, 1987; Marcus, 1990).

Parthenogenetic activation of oocytes by means of high voltage electric field pulses has become important in nuclear transplantation studies since the same electric pulses that fuse a nuclear donor cell with an enucleated oocyte can be used to artificially activate the ooplasm. However, electrical activation still remains one of the

least efficient steps in the nuclear transplantation procedure (Stice and Robl, 1988; Collas et al., 1989). The effect of electric pulses on oocyte activation has been reported in a variety of species, including the mouse (Tarkowski et al., 1970, Onodera and Tsunoda, 1989; Didion et al., 1990; Marcus, 1990), rabbit (Stice and Robl, 1988; Onodera and Tsunoda, 1989; Didion et al., 1990; Ozil, 1990; Collas and Robl, 1990), pig (Didion et al., 1990), sheep (Smith and Wilmut, 1989) and cow (Kono et al., 1989; Ware et al., 1989).

The exact mechanism by which electrofusion pulses are able to induce oocyte activation has not been determined. However, it has been proposed that when very short, high voltage pulses are applied to eukaryotic cell plasma membranes, a destabilization of the phospholipid bilayer occurs (reviewed by Zimmermann and Vienken, 1982). It is generally accepted that this reversible destabilization of the plasma membrane results in temporary formation of pores, which allow intracellular and extracellular exchange of ions and macromolecules (reviewed by Zimmermann and Vienken, 1982; Zimmermann et al., 1984). The formation of pores in the plasma membrane via electric pulse, also termed electroporation, has provided an effective means of allowing passage of ions as well as macromolecules such as DNA into eukaryotic cells (Chu, et al., 1987; Andreason and Evans, 1988).

The presence of Ca^{2+} ions in the pulse medium has been linked to rate of activation in mammalian oocytes receiving electric pulse treatment (Collas and Robl, 1990; Ozil, 1990). Recent reports propose a correlation between rate of electrical activation and influx of extracellular Ca^{2+} into the cytosol; indicating that changes in

ionic composition may play an important role in reinitiating meiotic events of electrically activated oocytes (Onodera and Tsunoda, 1989; Ozil, 1990).

We have begun to investigate the influence of extracellular Ca^{2+} and its effect on electric field mediated oocyte activation. The objectives of this study were two-fold: 1) to monitor electrofusion pulse induced $[Ca^{2+}]_i$ increase in murine oocytes placed in nonelectrolyte and electrolyte pulse media; and 2) to determine oocyte sensitivity to extracellular Ca^{2+} and its effect on oocyte activation. This paper reports dramatic changes in $[Ca^{2+}]_i$ levels after application of an electrofusion pulse, and, that increases in $[Ca^{2+}]_i$ directly influences rate of activation in electrofusion pulsed murine oocytes. In addition, oocyte membrane permeability to Ca^{2+} is shown to remain high for at least 5 min after electric pulse administration at room temperature.

MATERIALS AND METHODS

Oocyte Collection

Immature random bred Swiss (albino) female mice (day 23-30) were induced to superovulate by an injection of 7.5 IU pregnant mare serum gonadotropin (PMSG; Sigma Chemical Co., St.Louis, MO) followed 46-48 h later by an injection of 7.5 IU HCG (Sigma). Females were euthanised 17-18 h post-HCG and excised oviducts were placed into modified PB1 (Wood et al., 1987). Briefly, PB1 was made by adding 0.49 mM $MgCl_2 \cdot 6H_2O$, 0.33 Na pyruvate and 3 mg/ml bovine serum albumin (BSA, fraction V; Sigma) to phosphate buffered saline (PBS). Cumulus-oocyte complexes were removed from the oviduct by renting the distended ampulla and allowing complexes to be expelled from the oviduct. Cumulus cells were dispersed by gentle pipeting in PBS containing 0.1% hyaluronidase and 2 mg/ml polyvinylpyrrolidone (PVP, M_r 40,000;

Aldrich Chemical Company, Inc, Milwaukee, WI). Cumulus-free oocytes were washed three times in PB1 with morphologically abnormal oocytes (abnormal shape, size or dark pigmentation of ooplasm) being removed prior to random assignment to treatment groups. All manipulations and treatments were performed at room temperature (21°C, unless otherwise noted).

Ca^{2+} Indicator Loading of Oocytes

The Ca^{2+} indicator fluo-3 has been successfully used for fluorescent detection of $[Ca^{2+}]_i$ in a variety of cells (Kao et al., 1989; Minta et al., 1989). Fluorescent stain loading of oocytes was accomplished through use of the acetoxymethyl (AM) ester form of the long wavelength calcium indicator fluo-3 (Molecular Probes, Inc.,Eugene, OR). Cumulus free oocytes were incubated in 100 µl drops of PB1 containing 2 µM fluo-3/AM for 60 min at 37°C. Fluo-3 loaded oocytes were washed five times in PB1 to remove any extracellular fluo-3/AM and allowed to equilibrate to room temperature while preventing exposure to light. Loaded oocytes remained in darkness until initiation of treatment.

Treatment Media

Oocytes were randomly assigned to three nonelectrolyte and three electrolyte treatment media (table 1). Nonelectrolyte media consisted of 0.3 M mannitol (T1), 0.3 M mannitol + 0.05 mM $CaCl_2$ (T2) and 0.3 M mannitol + 0.9 mM $CaCl_2$ (T3). Electrolyte media consisted of Ca^{2+}-free PBS (T4), PBS containing 0.05 mM $CaCl_2$ (T5) and PBS containing 0.9 mM $CaCl_2$ (standard PBS solution, T6). Each oocyte was equilibrated in the assigned treatment medium with minimal exposure to light for 7 min prior to fluorescent measurement.

Table 1. Treatment Media and Resistance

Treatment	Media	Resistance (ohm)
T1	0.3 M mannitol	2.17×10^3
T2	0.3 M mannitol + 0.05 mM Ca^{2+}	1.75×10^3
T3	0.3 M mannitol + 0.9 mM Ca^{2+}	0.69×10^3
T4	Ca^{2+}-free PBS	32
T5	PBS + 0.05 mM Ca^{2+}	36
T6	PBS + 0.9 mM Ca^{2+}	43

Activation Pulse

Fluo-3 loaded and non-loaded oocytes were placed between the electrodes of a glass slide fusion chamber (400 μm distance between electrodes; BTX, Inc., San Diego, CA) which had been overlaid with 300 μl of fusion treatment media. A 5 sec, 3 volt alternating current (AC) alignment pulse followed by a single, square wave, direct current (DC) fusion pulse of 1.56 $kV \cdot cm^{-1}$ for 99 μsec was applied to the electrodes utilizing an Electro Cell Manipulator 200 (BTX). Pulse strength and duration as well as conductivity of the medium was monitored with the aid of a graphic pulse analyzer (Optimizer, BTX).

Monitoring Change in $[Ca^{2+}]_i$

A fusion chamber was placed on the mechanical stage of an inverted microscope (Nikon, Garden City, NY) which had been connected to a spectrofluorometer (SLM 8000, SLM Aminco, Urbana, IL). The fluo-3 excitation and emission wavelengths used throughout the experiment were 490 nm and 526 nm respectively. The intensity of the emitted light was measured by a photomultiplyer tube connected to the camera port of the inverted scope through a filter/shutter attachment. Corning glass bandpass filters (CS LC Number 3-70 and 3-73, Kopp Glass, Inc, Pittsburgh, PA) were used to decrease background photon interference. Fluorescence intensity measurements were acquired over a 416 sec time span, with integration readings of 1 sec duration obtained every 2 sec throughout the measurement period. A baseline fluorescent reading was established during the first 20 sec prior to electric pulse application. At 20 sec, a AC alignment pulse was initiated followed automatically 5 sec later by a DC electrofusion pulse. Change in fluorescence was continuously observed for an additional 391 sec.

Readings were monitored live as well as stored to computer disk for determination of $[Ca^{2+}]_i$. A total of 70 oocytes were used for fluorescent monitoring of changes in $[Ca^{2+}]_i$ (10/ treatment group, 10 no pulse control).

Rate of Oocyte Activation

Non-loaded oocytes used for the assessment of activation were randomly assigned to one of the previously mentioned electric pulse treatment groups. Oocytes were equilibrated and treated to electrical pulse in the manner described above, with the following exceptions; 1 to 5 oocytes were placed into the fusion chamber at a time and manipulations took place under normal fluorescent room lighting. Care was also taken to prevent fusion of the polar body to the oocyte by positioning the polar body-oolemma interface perpendicular to the electrodes. After treatment, oocytes remained in pulsing medium for approximately 2 min prior to being washed three times in 100 µl drops of Whitten's medium (Whitten and Biggers, 1968) which had been overlayed with silcone oil (Dimethylpolysoloxane, Wt=20 centistokes, Sigma) and equilibrated overnight at 37°C in an atmosphere of 5% CO_2 in air. Treated oocytes were then cultured 8 h in Whitten's medium prior to assessing rate of activation. Activation in this experiment was defined as the formation of one pronucleus and one polar body, two or more pronuclei, or two cells each containing a nuclear structure as observed by differential interference contrast (DIC) microscopy. A total of 718 oocytes were assessed for pronuclear formation.

Duration of Ca^{2+} Permeability

In order to assess the duration of cell membrane permeability to Ca^{2+}, fluo-3 loaded oocytes were equilibrated in the dark for 7 min in 0.3 M mannitol, then placed

into the fusion chamber containing 300 μl 0.3 M mannitol. Holding pipets were used to keep the oocyte in a fixed position throughout the measurement period. Pipets with a tip size of 80μm OD were connected to a 250μl screw syringe (Hamilton Inc, Reno NV) via 0.45 mm ID Tygon tubing. The syringe, tubing and pipet were filled with Fluorinert (Sigma) to provide a hydraulic system, which maintained gentle aspiration of the oocyte.

A 1.56 $kV \cdot cm^{-1}$ electrofusion pulse was applied at 20 sec as previously stated. The fluorescent reading was continuous until 316 sec post pulse, at which time the measurement was interrupted and 150 μl of the 300 μl in the fusion chamber was removed and replaced with 150 μl of 0.3 M mannitol + 1.8 mM $CaCl_2$, bringing the $[Ca^{2+}]$ of the fusion medium to approximately 0.9 mM. Interruption of fluorescent detection lasted for approximately 20 sec, after which detection resumed to completion at 416 sec. Control oocytes were placed in either 0.3 M mannitol or 0.3 M mannitol + 0.9 mM $CaCl_2$ and fluorescence was measured for 416 sec to establish lack of $[Ca^{2+}]_i$ increase and leakage of fluo-3 through the cell membrane.

Statistical Analysis

Repeated measures analysis of variance and Chi-square analysis was used to test differences in $[Ca^{2+}]_i$ and rate of activation, respectively. In addition, Bonferroni procedure was used in conjunction with least significant difference to conserve for type I error.

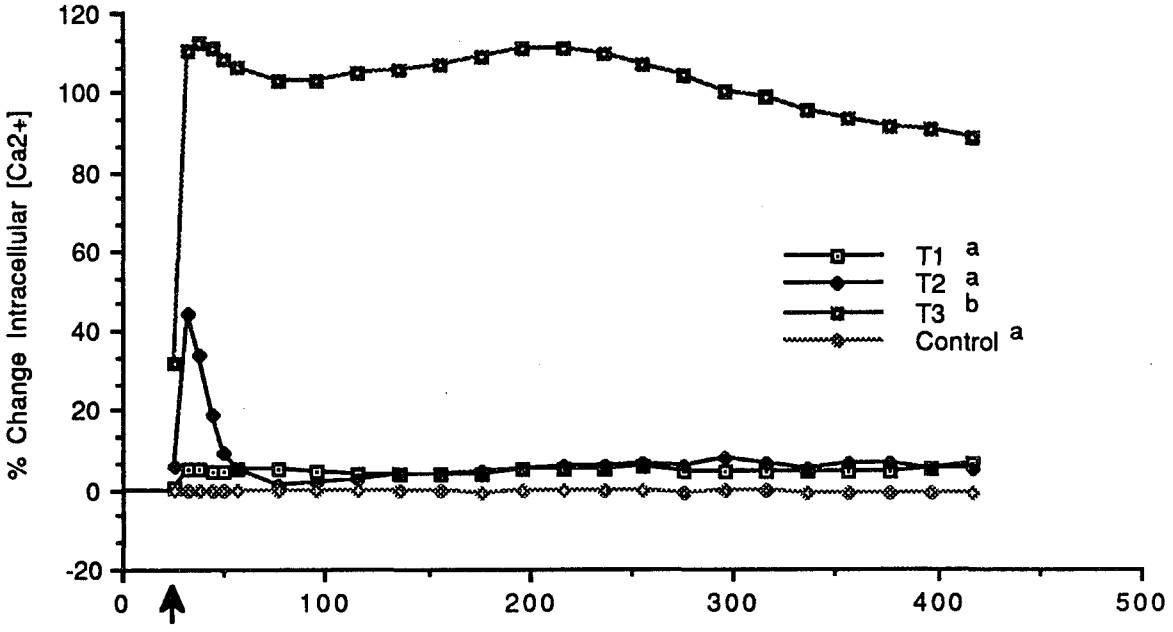
RESULTS

Electric Pulse Effect on Intracellular Ca^{2+} Levels

The change in $[Ca^{2+}]_i$ appeared to be directly related to the concentration of Ca^{2+} in the pulse medium. When cumulus free oocytes were subjected to an electrofusion pulse (1.56 $kV \cdot cm^{-1}$, 99 μsec) in nonelectrolyte (0.3 M mannitol) or electrolyte (PBS) media containing 0.9 mM Ca^{2+} an immediate and dramatic increase in $[Ca^{2+}]_i$ was detected (figures 1 and 2). In T3, $[Ca^{2+}]_i$ levels remained elevated, while T6 $[Ca^{2+}]_i$ levels decreased over time but never returned to baseline. A significant difference ($P<0.01$) in $[Ca^{2+}]_i$ levels for the entire period was observed between T3 and T6. There was no statistical difference in the peak $[Ca^{2+}]_i$ level 18 sec post pulse between groups T3 and T6 containing 0.9 mM Ca^{2+} ($P<0.05$). However, both T3 and T6 levels remained higher throughout the experiment than groups containing less than 0.9 mM Ca^{2+}. In addition, a significant difference was observed between pulse media containing 0.9 mM Ca^{2+} (T3, T6) compared to Ca^{2+}-free (T1, T4) and 0.05 mM Ca^{2+} (T2, T5) groups ($P<0.01$). Oocytes in T2 exhibited an initial rise in $[Ca^{2+}]_i$, however, the magnitude was significantly less than T3 and T6 (figures 1 and 2). Initial $[Ca^{2+}]_i$ rise in T2 was followed by a decline in intracellular levels to near baseline fluorescent readings at approximately 36 sec post electrofusion pulse. In T5, significant gradual increase in $[Ca^{2+}]_i$ was observed until the end of the 416 sec measurement period, at which time $[Ca^{2+}]_i$ levels equaled that observed 18 sec post pulse. No significant difference in overall $[Ca^{2+}]_i$ levels was detected between the 0.05 mM Ca^{2+} containing non-electrolyte (0.3 M mannitol) and electrolyte media ($P<0.05$).

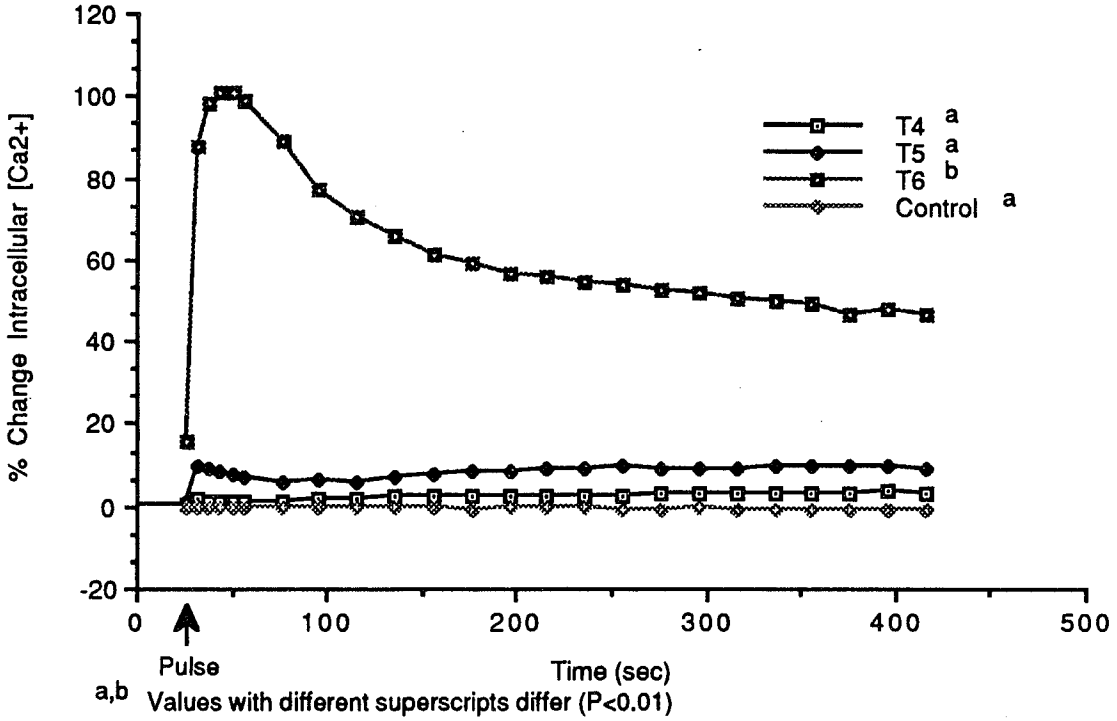
Oocytes treated in Ca^{2+}-free media (T1 and T4) exhibited a very slight rise in

Figure 1. Nonelectrolyte Fusion Medium (0.3 M Mannitol)



a,b Values with different superscripts differ (P<0.01)

Figure 2. Electrolyte Fusion Medium (PBS)



a,b Values with different superscripts differ (P<0.01)

$[Ca^{2+}]_i$ upon receipt of electrofusion pulse. Although the plots in figures 1 and 2 each illustrate the mean $[Ca^{2+}]_i$ of 10 oocytes, there were two oocytes from T4 which exhibited a secondary intracellular rise. Oocyte #2 in T4 exhibited two secondary $[Ca^{2+}]_i$ rises with the first beginning approximately 132 sec post pulse and lasting approximately 130 sec, and the second beginning approximately 312 sec and lasting for approximately 60 sec. Oocyte #6 exhibited a single secondary $[Ca^{2+}]_i$ rise beginning with a gradual rise at approximately 192 sec post pulse until 252 sec post pulse, at which time a rapid increase in $[Ca^{2+}]_i$ was observed.

Effect of Ca^{2+} Influx on Rate of Activation

The percent of murine oocytes activated by a 1.56 $kV \cdot cm^{-1}$, 99 μsec DC pulse was directly influenced by $[Ca^{2+}]$ and electrolyte makeup of the pulse medium (table 2). Maximum rates of activation were obtained when oocytes were pulsed in T3 (70.4%) or T6 (71.8%) treatment media. No significant difference in rate of activation was observed between T3 and T6 ($P>0.05$), and, both T3 and T6 exhibited significantly higher rates of activation than either T1, T2 or T4 ($P<0.05$). The increased rate of activation correlates with the increased extracellular $[Ca^{2+}]$ in T3 and T6. No difference ($P>0.05$) was observed between T1 (28.2%), T2 (31.1%) and T4 (35.6%). Rate of activation for the electrolyte medium T5 (57.3%) was significantly higher ($P<0.05$) than T1, T2 and T4, but significantly lower ($P<0.05$) than T3 and T6, indicating a possible synergistic effect of electrolyte medium and low concentrations of Ca^{2+}. These data demonstrate that although ionic strength of the medium may effect oocyte activation under certain electrofusion-electroporation conditions, the overriding factor is the presence of extracellular Ca^{2+} in the pulse medium.

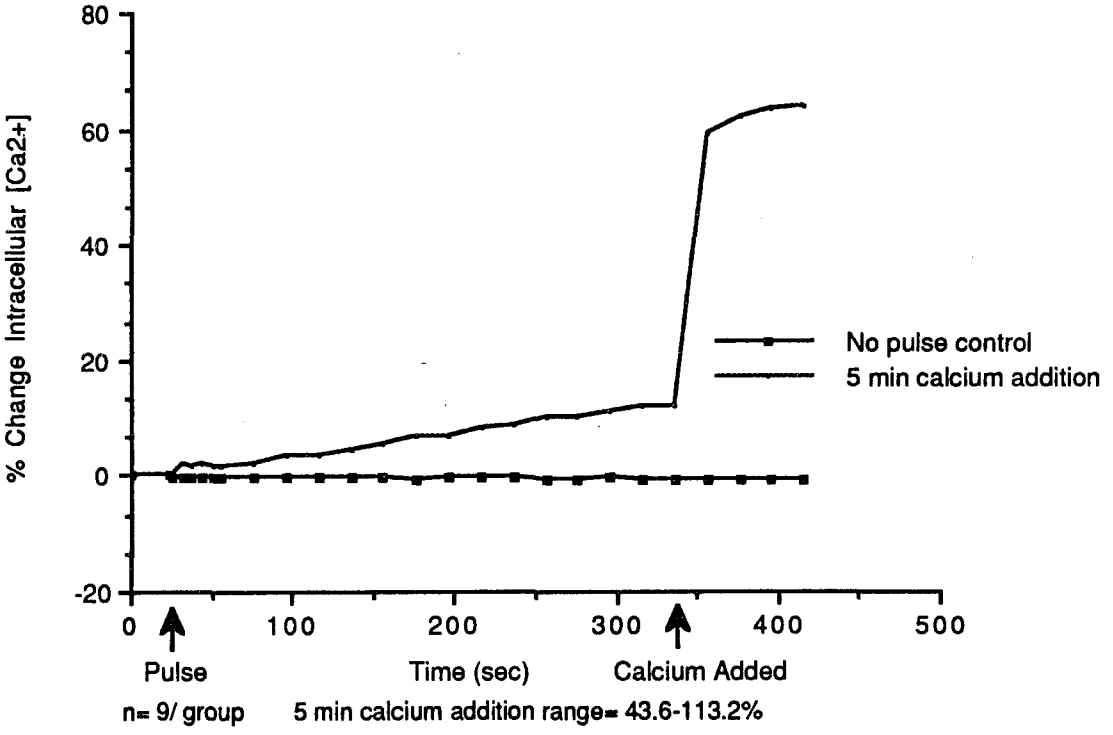
Table 2. Electric Pulse Induced Oocyte Activation

Treatment	No.	Pronuclei (%)
T1	103	29 (28.2)[c]
T2	103	32 (31.1)[c]
T3	98	69 (70.4)[a]
T4	104	37(35.6)[c]
T5	103	59(57.3)[b]
T6	103	74(71.8)[a]
Control (no pulse)	104	9 (8.7)[d]

[abcd] Different superscripts differ ($P<0.05$)
Data represents a total of 6 replicates

The presence of Ca^{2+} in the extracellular medium directly influenced rate of activation in murine oocytes. No significant difference in rate of activation was detected between nonelectrolyte and electrolyte media containing 0.9 mM Ca^{2+}. Our data (table 2) substantiates a previous report that isotonic culture media can be successfully used to activate murine oocytes (Onodera and Tsunoda, 1989). These data demonstrate the positive correlation of extracellular Ca^{2+} influx on electropulse activation of murine oocytes. In addition, oocytes pulsed in T1 medium at 21°C remained permeable to extracellular Ca^{2+} for a minimum of 5 min after receiving a 1.56 $kV \cdot cm^{-1}$ electrofusion pulse (figure 3).

Figure 3. Addition of 0.9 mM Calcium 5 min After DC Pulse



DISCUSSION

A variety of treatments and agents have been used to alter $[Ca^{2+}]_i$ and induce artificial activation in mammalian oocytes (reviewed by Kaufman, 1983). It has been demonstrated in the mouse that direct injection of Ca^{2+} into the ooplasm results in parthenogenetic activation (Fulton and Whittingham, 1978). Co-incubation with the Ca^{2+}, Mg^{2+} and H^+ ionophore A23187, causes release of Ca^{2+} from intracellular stores and induces oocyte activation in several species (Steinhardt et al., 1974; Ware et al., 1989). In addition, incubation in 7% ethanol is widely used as an effective activation procedure in the mouse (Kaufman, 1982; Cuthbertson, 1983) and has been shown to be effective in activating bovine oocytes (Nagai, 1987). It is accepted theory that high voltage DC electric field pulses causes reversible destabilization of the plasma membrane resulting in temporary depolarization and pore formation. This procedure provides an effective means of allowing passage of ions as well as macromolecules such as DNA across the plasma membrane and into the cytosol of eukaryotic cells (Chu et al., 1987; Andreason and Evans, 1988). The same membrane destabilization also induces fusion (termed electrofusion) of two cell membranes that are in direct cell to cell contact. Electrofusion is routinely used for hybridoma production and nuclear transplantation experiments (Zimmermann et al., 1985; Prather and First, 1990b). In addition, electroporation has been used to promote passage of extracellular Ca^{2+} across the acrosome of mammalian sperm and thereby inducing acrosome reaction (Tomkins and Houghton, 1988; Rickords et al., 1990).

In nuclear transplantation studies utilizing electrofusion, a DC pulse is applied with the intention of inducing two critical phenomena: 1) destabilize the plasma

membranes and induce cell-cell fusion; and 2) initiate release of enucleated oocytes from meiotic block at metaphase II. It has been postulated that membrane destabilization due to a single electric pulse may induce a single transient release of Ca^{2+} from intracellular stores, whereas multiple pulses may induce multiple releases of Ca^{2+} (Whittingham, 1980; Collas et al., 1989; Collas and Robl, 1990). Periodic increased in $[Ca^{2+}]_i$ have been reported to occur for at least 4 h following fertilization in the mouse (Cuthbertson and Cobbold, 1985).

Our data generated from microfluorometry experiments demonstrate that upon application of an electrofusion pulse to murine oocytes, substantial changes in $[Ca^{2+}]_i$ levels occur. Application of electric pulse significantly altered the membrane permeability to Ca^{2+} by inducing pore formation according to the accepted electrofusion-electroporation theory (Zimmermann and Vienken, 1982). When oocytes were pulsed in 0.3 M mannitol or PBS containing 0.9 mM Ca^{2+}, a dramatic increase in $[Ca^{2+}]_i$ was observed. In addition, an increase $[Ca^{2+}]_i$ level was maintained throughout the 6.5 min observation period.

Oocytes pulsed in Ca^{2+}-free PBS (T4, figure 2) exhibited a gradual (although not significant, $P>0.05$) increase in $[Ca^{2+}]_i$ over the observed time period. A possible explanation for this slight increase may be that the electrofusion pulse alone, or coupled with the stress induced from a Ca^{2+}-free environment, initiated low-level synthesis of the phosphoinositide pathway thereby producing small amounts of IP3 which causes release of Ca^{2+} from intracellular stores (Swann et al., 1987; Parker and Ivorra, 1990). In addition, environmental stress due to a lack of extracellular Ca^{2+} may have caused a gradual Ca^{2+} release from intracellular stores. A feedback mechanism

involving $[Ca^{2+}]_i$ acting upon intracellular stores and inducing Ca^{2+} release may also be implicated (Whittingham, 1980).

Oocytes pulsed in Ca^{2+}-free medium and subsequently placed into culture medium containing 0.9 mM Ca^{2+}, exhibited a rise in $[Ca^{2+}]_i$ levels (figure 3). The rise, however, did not raise the activation rate to that of oocytes pulsed in Ca^{2+} containing medium. This observation suggests that an immediate rise as well as a prolonged increase in $[Ca^{2+}]_i$ may be important in electrofusion mediated activation.

Many factors influence pore size and duration in mammalian cells (Tsong, 1983; Serpersu et al., 1985). One factor is the change in membrane potential induced by an electric pulse. It has been reported that a transmembrane potential of approximately 1 V is required to cause pore formation in phospholipid membranes (Zimmermann et al., 1984). When the field strength ($kV \cdot cm^{-1}$) exceeds that required to initiate pore formation, an increase in pore diameter and pore number is observed. The size and number of pores multiply in relation to field strength until membrane permeability becomes such that irreversible damage occurs, resulting in cell lysis (Zimmermann et al., 1985). Time duration of the pulse affects pore size as pore diameter increases in response to longer (nsec to μsec) pulse duration (Serpersu et al., 1985; Zimmermann et al., 1985). Other factors involved in formation of pore size include ionic strength of the medium (i.e., low ionic strength medium causes formation of larger pores) as well as cell type (Serpersu et al., 1985).

Time required for the resealing of electric pulse induced pores in lipid-protein membranes is also temperature dependant. When erythrocytes were maintained at 4°C, electric field induced permeability was shown to remain for up to 30 min after electric

field treatment (Zimmermann et al., 1980). Although electropores have been shown to be stable at 4°C, a rapid reduction in the size of the pore has been reported in some cell types (Serpersu et al., 1985). In contrast, cells maintained at 37°C throughout the electric pulse treatment were restored to their original impermeable state within a few minutes of pulse application (Zimmermann et al., 1980). Rapid closure of pores at higher temperatures is mainly due to greater fluidity of lipid and protein molecules in the membrane, depending on cell type peculiarities (Zimmermann et al., 1985). Erythrocyte permeability to Rb^+ at 37°C was maintained for 40 min when exposed to a 4 $kV \cdot cm^{-1}$, 2 μsec pulse, and as much as 20 h with a 3.7 $kV \cdot cm^{-1}$, 20 μsec pulse (Serpersu et al., 1985).

Maintenance of pore structure affects membrane fusion capability. Sowers (1986) reported that significant fusion yields in erythrocyte ghost cells were obtained at room temperature when cells were brought into membrane-membrane contact as much as 5 min post fusion pulse application. This experiment demonstrates that the applied DC pulse induced a long term fusogenic state, presumably by maintenance of fusogenic pores.

Our data (figure 3) demonstrate that murine oocyte membrane permeability to Ca^{2+} is maintained at room temperature in mouse oocytes for a minimum of 5 min post 1.56 $kV \cdot cm^{-1}$ DC pulse. Based on these data, it is possible that a threshold level of $[Ca2+]_i$ is required to activate murine oocytes using electrical activation. This hypothesis is supported by recent reports on rabbit oocyte activation (Ozil, 1990; Collas and Robl, 1990). Although the level of Ca^{2+} influx was not investigated, replacement of the 0.3 M glucose + 0.01 mM Ca^{2+} pulse medium with culture medium within

approximately 1 min did not appear to have an effect on rate of activation (Ozil, 1990). The author stated that rate of activation was directly correlated to pulse duration and presence of Ca^{2+} in the pulse medium. One important difference between the data reported by Ozil (1990) and data presented in this paper is that the former mentioned experiment was conducted at 38°C compared to 21°C as reported here. Our data (figure 3) demonstrate that electropulse induced membrane permeability to Ca^{2+} is maintained for at least 5 min when oocytes are kept at 21°C. In addition, by increasing $[Ca^{2+}]$ in the pulse medium to 0.9 mM, a significant increase in rate of activation of murine oocytes was observed.

CHAPTER IV

EFFECT OF ELECTROFUSION PULSE ON MURINE EMBRYOS SUBJECTED TO ELECTROLYTE AND NONELECTROLYTE FUSION MEDIUM

INTRODUCTION

Electric field mediated cell-to-cell fusion is commonly used to induce lipid membrane fusion between a variety of cell types. Electrofusion parameters are highly reproducible and provide researchers with the ability to fine tune electric field strengths in response to cells of differing size and/or species (Bates et al., 1987; Sun and Moor, 1989). Electric field strength can easily be regulated by monitoring conductance of the fusion medium and decreasing or increasing the voltage between the electrodes to ensure maximum survivability.

Electrofusion is currently used to fuse donor nuclei containing karyoplasts to enucleated cytoplasts in mammalian nuclear transplantation experiments. It has become common practice to place the karyoplast-cytoplast constructs in a low conductive, nonelectrolyte fusion medium (e.g. mannitol) during application of a high voltage direct current (DC) fusion pulse (Willadsen, 1986; Robl and Stice, 1989). Nonelectrolyte fusion media has been reported to provide a higher rate of fusion when an electrofusion pulse is applied to karyoplast-cytoplast constructs (Robl et al., 1987). It has also been reported that nonelectrolyte fusion media allows for greater embryonic viability post fusion pulse than electrolyte media (Robl and Stice, 1989). However, separate reports indicate the rate of fusion of 2-cell embryos is the same or slightly higher when a conductive electrolyte pulse medium (PBS) is used (Kubiak and Tarkowski, 1885; Tsunoda et al., 1987a). In the experiments using nonelectrolyte

medium, an alternating current (AC) alignment pulse was applied prior to the DC fusion pulse. In the electrolyte experiments, only a DC fusion pulse was applied. Nonconductive media has been used when an AC alignment pulse is applied to polarize the cells and bring cells within close proximity into direct membrane contact (Zimmermann and Vienken, 1982). The rationale for using nonconductive fusion media in nuclear transplantation is to prevent conductive heat buildup which can damage early embryonic cells (Robl et al., 1987).

This discrepancy over electrolyte concentration in fusion media prompted a study to determine whether the ionic composition used in our micromanipulation medium (PB1, Wood et al., 1987) could be used in conjunction with an AC alignment pulse followed by a DC fusion pulse.

Previous experiments have shown electrolyte medium (i.e. PBS) to have a positive effect on electric pulse induced murine oocyte activation (Chapter III). In addition, these results also indicated pulse media containing 0.9 mM Ca^{2+} induced a dramatic increase in the rate of murine oocyte activation compared to oocytes pulsed in media containing 0.0 or 0.05 mM Ca^{2+}. Based on these results, the present study was designed to determine what effect these electropulse parameters would have on rate of fusion, lysis and embryo viability. The present study was designed to: 1) determine the effect of nonelectrolyte (0.3 M mannitol) and electrolyte (PBS) pulse media on rate of fusion of murine 2-cell embryos, and 2) determine viability of murine pronuclear stage embryos subjected to a 3 V, 5 sec AC alignment pulse followed by a single 1.56 $kV \cdot cm^{-1}$ electrofusion pulse applied in nonelectrolyte 0.3 M mannitol and electrolyte PBS media.

MATERIALS AND METHODS

Embryo Collection

Prepubertal randomly bred Swiss albino female mice (day 23-30) were induced to superovulate as described in Chapter III. HCG injected females were immediately placed with fertile Swiss albino males and successful mating was determined by presence of a copulatory plug 16-17 h post HCG. Excised oviducts were placed into PB1 medium and pronuclear stage embryos were obtained 18-20 h post HCG by renting the ampulla and allowing the zygotes to be expelled. Cumulus cells were dispersed by gentle pipeting in PBS containing 0.1% hyaluronidase and 2 mg/ml polyvinylpyrrolidone (PVP, M_r 40,00; Aldrich Chemical Company, Inc, Milwaukee, WI). Collection of 2-cell embryos was performed by excising oviducts 44-46 h post HCG and flushing the oviducts with PB1 using a 30 gauge needle. All embryos were washed three times in PB1 prior to being randomly assigned to individual treatment groups. Pronuclear and 2-cell embryos were held in PB1 until placement into culture. Pronuclear stage nuclear transfer controls were held in micromanipulation medium (see below) prior to culture.

Treatment Media

Embryos were randomly assigned to three nonelectrolyte and three electrolyte treatment media. Nonelectrolyte media consisted of 0.3 M mannitol (T1), 0.3 M mannitol + 0.05 mM CaCl2 (T2) and 0.3 M mannitol + 0.9 mM CaCl2 (T3). Electrolyte media consisted of Ca^{2+}-free PBS (T4), PBS containing 0.05 mM $CaCl_2$ (T5) and PBS containing 0.9 mM $CaCl_2$ (T6).

Electrofusion

A glass slide fusion chamber (BTX, Inc., San Diego, CA), made of two stainless steel wire electrodes positioned 400 μm apart and overlaid with 300 μl of fusion medium, was used for all electrofusion treatments. Embryos were placed centrally between the electrodes and manually aligned to orient the cell-cell membrane contact parallel to the electrodes. A 3 V, 5 sec alternating current (AC) dielectrophoretic pulse, followed by a single 1.56 $kV \cdot cm^{-1}$, 99 μsec direct current (DC) fusion pulse was applied to the electrodes using a high voltage pulse generator (Electro Cell Manipulator 200, BTX). Duration and strength of the pulse was monitored by a graphic pulse analyzer (Optimyzer, BTX). Rate of fusion on 2-cell embryos subjected to electrofusion was assessed 2 h after the pulse.

Micromanipulation

Pronuclear stage embryos were incubated in PB1 containing cytoskeleton inhibitor (7.5 μg/ml cytochalasin B, Sigma) 30 min prior to and throughout micromanipulation. Nuclear transplantation was performed as described by McGrath and Solter (1983) with a few modifications. Briefly, a beveled and sharpened micropipet (21-23 μm tip OD) was used to remove the two pronuclei along with a small amount of cytoplasm encased in plasma membrane (karyoplast). The pronuclei containing karyoplast was then inserted into the perivitelline space of a previously enucleated zygote (cytoplast). Karyoplast-cytoplast constructs were placed into assigned fusion medium (T1, T3 and T6) and an electrofusion pulse was applied as previously described.

Embryo Culture

All pronuclear embryos were placed into 100 µl drops of CZB medium (Chatot et al., 1989) which had been overlayed with dimethylpolysoloxane (Wt=20 centistokes, Sigma) and equilibrated overnight at 37°C in a humidified atmosphere of 5% CO_2 and air. After 44 h of culture, embryos were evaluated for development to the 4-cell stage. At this time, all embryos developing to the 4-cell stage were removed from CZB and placed into 100 µl drops of Whitten's medium (Whitten and Biggers, 1968) under dimethylpolysoloxane. Embryos were cultured for an additional 54 h at which time the number developing to blastocyst stage was assessed.

Treatments

In experiment 1 the objective was to determine the rate of fusion and rate of lysis in murine 2-cell embryos placed in nonelectrolyte (0.3 M mannitol; T1-T3) and electrolyte (PBS; T4-T6) fusion medium and subjected to a fusion procedure of a 3 V, 5 sec AC alignment pulse, followed by a 1.56 $kV \cdot cm^{-1}$, 99 µsec DC fusion pulse. Control 2-cell embryos were placed in T1 for 2 min and did not receive a fusion pulse.

The objective of experiment 2 was to evaluate the development of pronuclear stage embryos to the blastocyst stage in vitro after receiving the fusion pulse in T1, T3, and T6. Control embryos were not subjected to fusion treatment.

In experiment 3, T3 and T6 were used to test the rate of fusion and rate of development on pronuclear stage karyoplasts fused to enucleated pronuclear stage cytoplasts.

Statistical Analysis

Embryonic development to a specific cell stage was analyzed by Chi-square analysis.

RESULTS

Electrolyte vs Nonelectrolyte Fusion Media

Table 3 summarizes the rate of fusion of 2-cell stage embryos placed into treatment media (T1-6) and subjected to a 3 V, 5 sec AC alignment pulse followed by a single 1.56 $kV \cdot cm^{-1}$ DC fusion pulse. Embryos pulsed in T5 and T6 exhibited significantly higher rates of fusion (96.9 and 92.9%, respectively) compared to T1 (83.7%), T2 (84.7%) or T3 (77.6%) ($P<0.05$). There was no significant difference ($P>0.05$) in rate of lysis between any of the treatment groups. Control embryos did not exhibit any lysis. Although rate of lysis remained low throughout the experiment, 5/98 (5.1%) exhibited lysis in T3 compared to 1/98 (1.0%) in T6 and 0/98 (0.0%) in T1, T2, T4, and T5.

In Vitro Development of Pronuclear Stage Embryos

Once rate of fusion and lysis of murine 2-cell embryos had been identified, we evaluated the effect of T1, T3 and T6 on pronuclear development in vitro. Table 4 summarizes the rate of development of pronuclear stage embryos placed in T1, T3 and T6 and subjected to the previously described electrofusion procedure. In vitro culture to 44 h resulted in 68.7, 65.4, 73.2 and 67.5% of the pronuclear stage zygotes reaching the 4-cell stage for T1, T3, T6 and control embryos, respectively. Culture to 98 h after fusion pulse resulted in 55.5, 52.3, 50.0 and 54.2% of T1, T3, T6 and control embryos developing to the blastocyst stage. There was no significant difference ($P>0.05$)

Table 3. Rate of Fusion of 2-Cell Murine Embryos Subjected to a 3 V, 5 sec AC, 1.56 $kV \cdot cm^{-1}$ DC Electrofusion Pulse

Group	N	Fused(%)	Lysis(%)
T1	98	82(83.7)^{ab}	0(0.0)
T2	98	83(84.7)^{ab}	0(0.0)
T3	98	76(77.6)^{a}	5(5.1)
T4	98	86(87.8)^{bc}	0(0.0)
T5	98	95(96.9)^{d}	0(0.0)
T6	98	91(92.9)^{cd}	1(1.0)
Control (No Pulse)	98	0(0)^{e}	0(0)

^{abcde} Different superscripts differ ($P<0.05$)
Data represents a total of 5 replicates

Table 4. In Vitro Development of Pronuclear Stage Embryos Subjected to Electrofusion Pulse

Group	N	Lysis(%)	4-Cell(%) at 44 h	Blast(%) at 98 h
T1	99	1(1.0)	68(68.7)[a]	53(55.5)[a]
T3	107	1(0.9)	70(65.4)[a]	56(52.3)[a]
T6	112	0(0.0)	82(73.2)[a]	56(50.0)[a]
Control (No Pulse)	120	0(0.0)	81(67.5)[a]	65(54.2)[a]

[a] No significant difference ($P>0.05$)
Data represents a total of 5 replicates

between treatment groups in development to the blastocyst stage after 98 h in culture.

In Vitro Development Involving Pronuclear Transfer

Two treatment media, T3 and T6 were used for electrofusion treatment to fuse pronuclear karyoplast-cytoplast complexes and determine their rate of development to the blastocyst stage in vitro. The T3 and T6 media were selected on the basis that no detrimental effect was observed in treated pronuclear stage embryos (table 4) and previous experiments demonstrated an increase in rate of murine oocyte activation when unfertilized oocytes were electrically activated in T3 and T6 (Chapter III). No significant difference (table 5) was observed in the rate of pronuclear karyoplast-cytoplast fusion between T3 (79.7%) and electrolyte T6 (85.5%) media ($P>0.05$). There was also no difference in the rate of development to the 4-cell stage (78.0 vs 72.9%) and blastocyst stage (59.3 vs 54.2%; T3 and T6 respectively) ($P>0.05$). However, a difference was observed in rate of development to the 4-cell stage and blastocyst stage between non-pulsed control pronuclear stage embryos and T6 treated karyoplast-cytoplast constructs (86.3 vs 72.9% and 68.5 vs 54.2%, respectively) ($P<0.05$). In addition, there was no difference in the rate of lysis observed between T3, T6 and control pronuclear stage embryos.

Table 5. In Vitro Development of Pronuclear Transplanted Embryos

Group	N	Fused	Lysis(%)	4-Cell(%)	Blast(%)
T3	74	59(79.7)	1(1.7)	46(78.0)[ab]	35(59.3)[ab]
T6	69	59(85.5)	2(3.4)	43(72.9)[a]	32(54.2)[a]
Control (No Manipulation)	73	--	0(0.0)	63(86.3)[b]	50(68.5)[b]

[ab] Different superscripts differ ($P<0.05$)
Data represents a total of 4 replicates

DISCUSSION

Cell-to-cell fusion is obtained when two or more lipid bilayer membranes maintained in direct contact are disrupted to the point where reanealing of the adjacent membranes results in the formation of a single cell. Cell fusion is usually achieved in vitro by disrupting phospholipid membranes through use of chemicals (Baker and Kalra, 1979; Majumdar et al., 1980), fusogenic viruses (Knutton, 1978; Lucy, 1982), or application of electric fields (Zimmermann and Vienken, 1982). Electric field mediated membrane fusion has become popular due to its ease of use and high reproducibility.

The electric field induced cell fusion procedure used in nuclear transplantation experiments has routinely been performed by placing karyoplast-cytoplast constructs in a nonelectrolyte fusion medium such as mannitol during application of the high-voltage electrofusion pulse (Willadsen, 1986; Robl et al., 1987; Robl and Stice, 1989). The rationale for using nonelectrolyte media originated from electrofusion experiments involving erythrocytes (Scheurich and Zimmermann, 1981) and sea urchin eggs (Richter et al., 1981). To alleviate the need for high cell densities or chemical pretreatment to provide sufficient cell-to-cell membrane contact, a nonuniform AC electric field was applied prior to the DC fusion pulse. The nonuniform AC field induces cell-cell membrane contact by polarizing the individual cells. This formation of dipoles causes cells in close proximity to orient themselves towards the oppositely charged dipolar region of adjacent cells and are drawn into direct membrane contact (Scheurich and Zimmermann, 1981; Zimmermann et al., 1981). This phenomenon, termed dielectrophoresis (Pohl, 1978), is used to align cells into pearl chains with resulting membrane-membrane contact remaining parallel to the electrodes. The

dielectrophoretic AC alignment pulse applied prior to induction of the DC fusion pulse greatly improves the rate of fusion in cell suspensions (Zimmermann et al., 1984).

Media used in electrofusion experiments have largely resulted from procedures utilizing an AC dielectrophoretic pulse to align cells into pearl chains (Richter et al., 1981). The dielectrophoretic alignment pulse is generally performed in low conductive, nonelectrolyte solutions made up of isotonic concentrations of sugars (Zimmermann et al., 1984). The NaCl concentrations found in cell culture media is replaced by isosmotic concentrations of mannitol, glucose, sucrose or sorbitol to maintain osmotic pressure while decreasing the conductivity of the pulse medium (Zimmermann and Vienken, 1982). When an AC pulse passes through a conductive electrolyte medium such as PBS, localized heating occurs and turbulence is produced. When the resistance of the pulse medium exceeds $10^{-4}\ \Omega \cdot cm^{-1}$, convective heat currents arise during the cell alignment AC pulse and disrupts pearl chain formation and resulting cell-cell contact (Zimmermann et al., 1985). Therefore, isosmotic solutions of low conductive nonelectrolytes are used to maintain internal osmotic pressure while reducing possible detrimental effects associated with the heating of fusion medium (Bates et al., 1987).

Since nuclear transfer experiments do not involve the formation of pearl chains of multiple cells, the use of nonelectrolyte media is not required for successful membrane fusion. Kubiak and Tarkowski (1985) reported successful fusion was obtained when DC fusion pulses were applied to 2-cell mouse embryos placed in either 0.3 M mannitol or PBS. Subsequent development of the tetraploid embryos to the blastocyst stage in vitro suggested no difference in embryonic viability. Tsunoda et al., (1987a) reported the successful fusion of pronuclear karyoplast-cytoplast complexes

placed in PBS fusion medium. The proportion of pregnant recipients and offspring produced after fusion treatment did not differ between electrofusion in electrolytic PBS or viral induced fusion by inactivated Sendai virus treatments. In both reports by Kubiak and Tarkowski (1985) and Tsunoda et al., (1987a) the membrane-membrane fusion plane was manually aligned parallel to the electrodes and only a DC fusion pulse was applied. In contrast, Robl et al., (1987) reported nonelectrolyte Zimmermann Cell Fusion medium gave a higher fusion rate than electrolyte TL Hepes medium when ooplasm cytoplasts were fused to bovine oocytes, however, the numbers involved were low (8/9 and 1/9 respectively). Based on their results as well as previous reports using glucose and mannitol (Ozil and Modlinski, 1986; Willadsen, 1986), the use of nonelectrolyte fusion media has become standard in mammalian nuclear transplantation experiments.

We have occasionally observed unexplained cell lysis immediately after micromanipulated karyoplast-cytoplast constructs were placed in 0.3 M mannitol fusion medium, even though the medium had been adjusted to a pH of 7.2 and maintained an osmolarity of 280 mosm. At the time, constructs kept in manipulation medium did not lyse until they, too, were placed into the fusion medium. One explanation for this observed effect is the stress placed upon the membranes during the enucleation and transfer of nuclei may have been sufficient to cause cell lysis once the constructs were removed from the physiologically balanced electrolyte manipulation medium (PB1) and placed into nonelectrolyte fusion medium (0.3 M mannitol). It may be advantageous to place the constructs into a physiologically balanced fusion medium (i.e. PB1) to decrease the ionic stress placed upon the manipulated cell membranes (Zimmermann

et al., 1985; Bates et al., 1987). In addition, it may be important to consider the ionic composition of the cytosol in order to prevent detrimental ion concentration gradients that may occur after electric pulse induced membrane pore formation (Zimmermann et al., 1984). Electrofusion media containing an ionic concentration too low or too high may directly effect post fusion viability by subjecting the cytosol to possible deleterious effects of ionic stress.

Here we report the application of a 3 V, 5 sec AC alignment pulse prior to a single 1.56 $kV \cdot cm^{-1}$ DC fusion pulse to electrolyte PBS which results in successful fusion of murine 2-cell blastomeres at a rate equal to that of nonelectrolyte 0.3 M mannitol (table 3). In vitro development of pronuclear stage (table 4) or fused pronuclear karyoplast-cytoplasts (table 5) demonstrate that an AC alignment pulse followed by a DC fusion pulse does not adversely effect early murine embryonic development in vitro. Preliminary experiments involving sheep and goat nuclear transplantation indicate a high rate of fusion is obtained with the above mentioned fusion protocol (unpublished observations). Preliminary results evaluating embryonic development to term indicate no detrimental effects of PBS fusion medium. However, definitive data on rate of development remains to be obtained. We conclude that a 3 V, 5 sec AC alignment pulse can be applied prior to a 1.56 $kV \cdot cm^{-1}$ DC fusion pulse in electrolyte fusion medium (PBS) and no difference was observed in the in vitro development of pronuclear stage embryos pulsed in either 0.3 M mannitol containing 0.9 mM Ca^{2+} or PB1.

CHAPTER V

ELECTROPORATION OF INOSITOL 1,4,5-TRIPHOSPHATE INDUCES REPETITIVE CALCIUM OSCILLATIONS IN MURINE OOCYTES

INTRODUCTION

Upon sperm-egg fusion a dramatic change in physiological events takes place within the fertilized egg. Striking changes in membrane potential and $[Ca^{2+}]_i$ have been observed in various invertebrate and vertebrate species (Cuthbertson et al., 1981; Epel, 1982; Jaffe, 1983). One of the first events observed in the fertilized egg is a propagating Ca^{2+} wave that begins at the fertilization site and migrates through the cytoplasm to the opposite pole (Jaffe, 1985; Swann and Whitaker, 1986). The propagating wave and its corresponding rise in $[Ca^{2+}]_i$ triggers cortical granule exocytosis and initiates events leading to egg activation, defined as the resumption of second meiosis and second polar body formation (Jaffe, 1985; Miyazaki et al., 1986). In the sea urchin, sperm-egg fusion causes a change in membrane potential which is important for an immediate electrical block to polyspermy. A subsequent increase in $[Ca^{2+}]_i$ induces cortical granule exocytosis and development of the fertilization envelope (permanent block to polyspermy; Jaffe, 1985). In mammalian eggs, membrane potential changes do not elicit an electrical block to polyspermy (Miyazaki and Igusa, 1982; Jaffe, et al., 1983; McCulloh et al., 1983). However, changes in membrane potential are observed and are manifested as recurring transient hyperpolarization responses (Miyazaki and Igusa, 1981; Igusa et al., 1983; McCulloh et al., 1983). These hyperpolarization responses initiated at fertilization are induced by a Ca^{2+} activated K^+ conductance and are elicited by periodic transient oscillations in $[Ca^{2+}]_i$ (Igusa and

Miyazaki, 1986). In the fertilized mammalian egg, periodic transient $[Ca^{2+}]_i$ oscillations occur after sperm-egg fusion and these oscillations have been reported to occur for at least 4 h in the mouse (Cuthbertson and Cobbold, 1985) and at least 30 min in the hamster (Miyazaki et al., 1986). Maintenance of these recurring oscillations is due to periodic Ca^{2+} release from intracellular stores (Cuthbertson and Cobbold, 1985; Igusa and Miyazaki, 1986).

It has long been established that increasing the cytosolic $[Ca^{2+}]$ in oocytes causes parthenogenetic activation. Various agents and methods used in parthenogenetic activation, including ethanol (Colonna et al., 1989), calcium ionophores (Cuthbertson et al., 1981), and microinjection of Ca^{2+}, elicit a rapid rise in $[Ca^{2+}]_i$. However, these methods have not been shown to induce a series of transient $[Ca^{2+}]_i$ oscillations as those observed to occur in fertilized eggs. A recent study involving parthenogenetic activation in rabbit oocytes reported a method designed to mimic prolonged $[Ca^{2+}]_i$ oscillations (Ozil, 1990). Repeated increases in $[Ca^{2+}]_i$ levels were stimulated by applying a 1.8 $kV \cdot cm^{-1}$ electric pulse every 4 min for a duration of 1.5 h. The 22 pulse treatment resulted in significant increase in rate of development beyond the third cell cycle. It was concluded that the process of oocyte activation is not a time limited event and therefore repeated increases in $[Ca^{2+}]_i$ may be important in turning on critical cell processes necessary for normal embryonic development.

The mechanism by which sperm-egg fusion induces hyperpolarizing responses and prolonged $[Ca^{2+}]_i$ oscillations remains unclear. One hypothesis is that sperm-egg binding activates receptors affiliated with GTP-binding proteins (G proteins) which in turn activate the phosphoinositide cascade resulting in an increase in the intracellular

second messenger inositol 1,4,5-triphosphate (IP3) (Miyazaki, 1988b). The IP3 acts on the endoplasmic reticulum to induce release of stored Ca^{2+} by binding to a receptor, thus opening channels for stored Ca^{2+} to exit into the cytosol. A second hypothesis suggests that IP3 triggers the initial release of Ca^{2+} from IP3 sensitive stores and this release of stored Ca^{2+} induces the $[Ca^{2+}]_i$ oscillations by functioning as a positive regulator for Ca^{2+} induced Ca^{2+} release (CICR) from IP3 insensitive stores (Igusa and Miyazaki, 1983; Berridge, 1988).

Recently repetitive $[Ca^{2+}]_i$ oscillations that mimic those observed in fertilized oocytes have been elicited in hamster oocytes through microinjection of a sperm factor obtained from hamster and porcine sperm (Swann, 1990). This argues against the model that sperm binding to a receptor-G-protein system induces the repetitive $[Ca^{2+}]_i$ oscillations. Instead, the microinjected sperm factor argues for the model of CICR in propagating $[Ca^{2+}]_i$ oscillations.

Here we report electroporation of IP3 into the cytosol of murine oocytes causes a dramatic rise in $[Ca^{2+}]_i$ resulting from Ca^{2+} release from intracellular stores as well as periodic oscillations in $[Ca^{2+}]_i$ that persist for a minimum of 20 min in Ca^{2+}-free medium. In addition, it was demonstrated that addition of ethanol (ETOH) induces a dramatic release of Ca^{2+} from intracellular stores.

MATERIALS AND METHODS

Collection, loading of fluorescent Ca^{2+} indicator fluo-3, and fluorescent intensity measurement was accomplished as desribed in chapter III with the exeption that observation time was extended up to 1248 sec.

Treatment of Oocytes

The experimental design consisted of comparing the the rise in $[Ca^{2+}]_i$ of fluo-3 loaded oocytes subjected to electroporation in PBS and Ca^{2+}-free PBS, each containing 25 μM IP3, to that elicited by PBS and Ca^{2+}-free PBS containing a final concentration of 7% ETOH. Non-pulsed control oocytes were placed in PBS+ 25 μM IP3 during monitoring of $[Ca^{2+}]_i$ fluorescence.

Electroporation of IP3 was accomplished by placing fluo-3 loaded oocytes in the electrofusion chamber and treated with an electric pulse as described in chapter III. Equilibrated oocytes were then placed into the electroporation chamber containing 300 μl PBS + 25 μM IP3 or Ca^{2+}-free PBS + 25 μM IP3.

For ETOH treatment, fluo-3 loaded oocytes were placed in PBS or Ca^{2+}-free PBS and allowed to equilibrate for 7 min in the dark. No pulse was applied to ETOH treatment oocytes. Micropipets with a tip size of 80 μm OD were used to keep the oocyte in a fixed position throughout the measurement period. Pipets were connected to a 250 μl screw syringe (Hamilton Inc, Reno, NV) via 0.45 mm ID Tygon tubing. The syringe, tubing and pipet were filled with Fluorinert (Sigma) to provide a hydraulic system, which maintained gentle aspiration of the oocyte. After a 20 sec baseline fluorescent reading was obtained, fluorescent measurement was interrupted and 150 μl of PBS (or Ca^{2+}-free PBS) was removed. The removed media was replaced with 150 μl of 14% ETOH in PBS (or Ca^{2+}-free), bringing the final concentration after equilibration to 7% ETOH. Fluorescent intensity measurement resumed immediately following the addition of 14% ETOH.

RESULTS

Electroporation of IP3

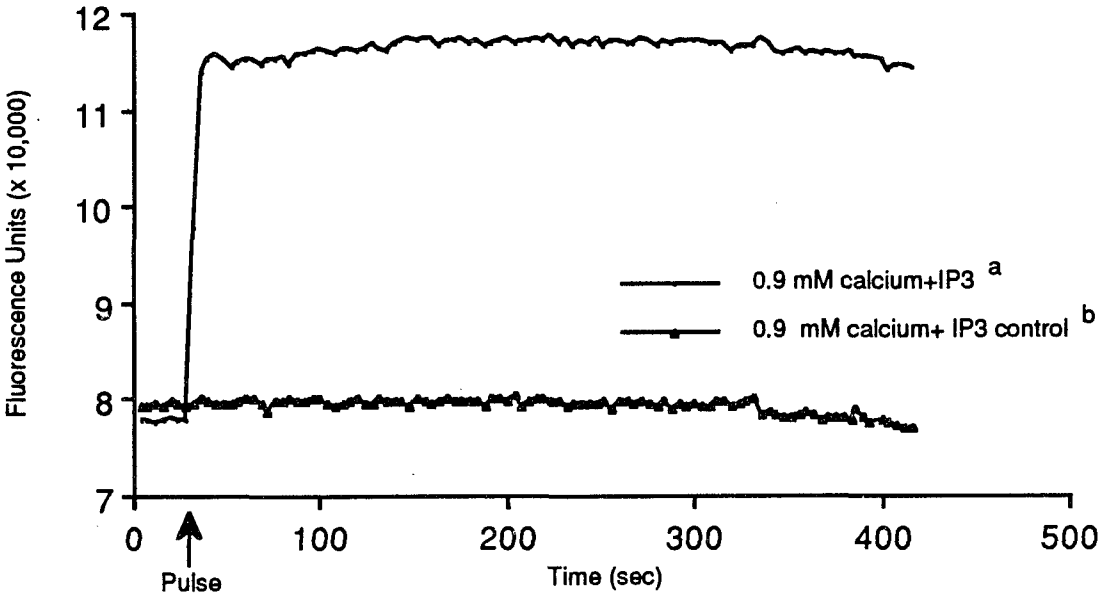
Figure 4 depicts results obtained when murine oocytes were electroporated in PBS +25 μM IP3. A dramatic and immediate rise in $[Ca^{2+}]_i$ was observed upon application of 3V, 5 sec AC, 1.5 $kV \cdot cm^{-1}$, 99 μsec electroporation pulse and $[Ca^{2+}]_i$ was maintained at an elevated level for a min of 14 min. The presence of extracellular Ca^{2+} kept measured $[Ca^{2+}]_i$ levels elevated and masked any potential periodic Ca^{2+} release from intracellular stores that may have occurred due to electroporated IP3.

Figure 5 illustrates repetitive $[Ca^{2+}]_i$ oscillations in a representative mouse oocyte generated by electroporation of 25 μM IP3 in Ca^{2+}-free PBS. An immediate increase in $[Ca^{2+}]_i$ is observed upon application of the electroporation pulse, followed by a series of $Ca^{2+}{}_i$ oscillations that occurred for 20.5 min. A gradual increase in the interval between $[Ca^{2+}]_i$ oscillation peaks occurred over time in Ca^{2+}-free PBS + 25 μM IP3. The $[Ca^{2+}]_i$ oscillation intervals ranged from 0.5 to 3 min. Similar responses were observed in additional oocytes with intervals ranging from 0.3 to 5 min.

ETOH Induced Rise in $[Ca^{2+}]_i$

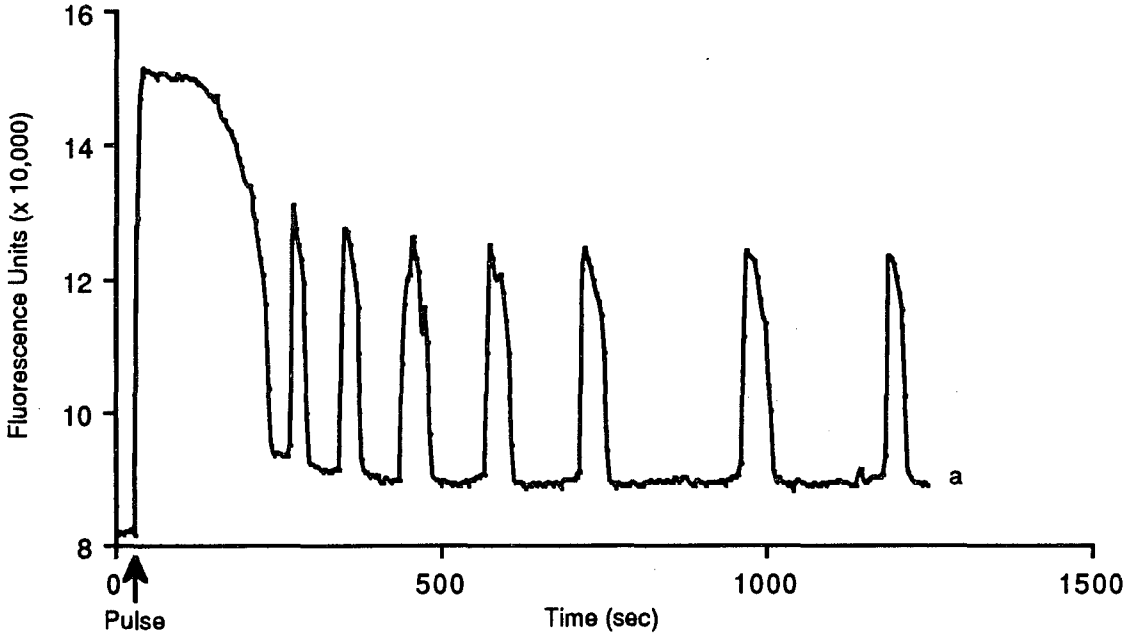
Figure 6 illustrates the dramatic rise in mouse oocyte $[Ca^{2+}]_i$ upon treatment with 7% ETOH in PBS and Ca^{2+}-free PBS. There was no significant difference ($P>0.05$) in $[Ca^{2+}]_i$ between PBS + ETOH and CA^{2+}-free PBS + ETOH indicating the rise in $[Ca^{2+}]_i$ resulted from a release of Ca^{2+} from intracellular stores.

Figure 4. Electroporation of Murine Oocytes in PBS + 25 μM IP3



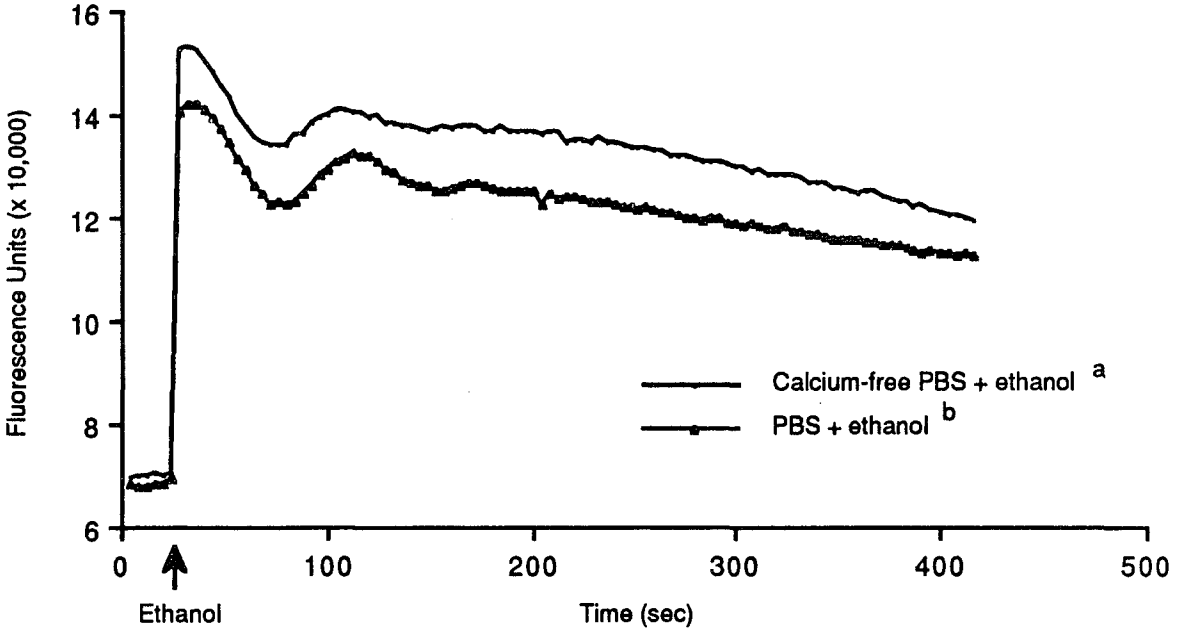
a,b Represetative sample of individual oocytes
n= 8 oocytes/group
0.9 mM calcium + IP3 range= 8.6-13.1 x 10,000 fluorescence units

Figure 5. Electroporation of Murine Oocyte in Calcium-Free PBS + 25 μM IP3



[a] Representative sample of an oocyte measured for 20.5 min

Figure 6. Ethanol Induced Intracellular Calcium Release



a,b Representative sample of single oocytes

Calcium-free PBS + ethanol: n=5, range 54.28-155.34 Rel %
PBS + ethanol: n=6, range 41.97-145.60 Rel %

DISCUSSION

Inositol 1,4,5-triphosphate (IP3) is a component of the intracellular phosphoinositide second messenger system and acts by binding to a specific receptor on the endoplasmic reticulum. The IP3-receptor binding induces opening of specific Ca^{2+} channel proteins, which in turn allow Ca^{2+} ions to exit the internal store and enter the cytosol. IP3 has been shown to induce an intracellular release of Ca^{2+} in a variety of cell types (Berridge and Irvine, 1984). The rise in $[Ca^{2+}]_i$ is part of a biological signal transduction process that controls such cell functions as proliferation, metabolism and secretion (Reviewed by Epel, 1978; Berridge and Irvine, 1984; Jaffe, 1985). The IP3 second messenger system has been implicated in triggering critical events in oocyte activation (Whitaker, 1989; Whitaker and Patel, 1990) as well as in the initiation of DNA synthesis, either alone or in conjunction with diacylglycerol (Epel, 1990).

Research conducted on oocytes indicates that IP3 induces intracellular Ca^{2+} release in amphibians (Busa et al., 1985; Han and Nuccitelli, 1990), sea urchin (Whitaker and Irvine, 1984; Turner et al., 1986; Steinhardt and Alderton, 1988) and hamster (Miyazaki, 1988b). A single injection (2 nM cytoplasmic volume) of IP3 into the hamster ooplasm induces an immediate rise in $[Ca^{2+}]_i$ with a duration of 13-18 sec (Miyazaki, 1988b). The single injection of IP3 was reported to produce only one $[Ca^{2+}]_i$ rise while continuous leakage of IP3 from the injection pipette produced a series of $[Ca^{2+}]_i$ transients similar to that observed when the oocyte was fertilized by sperm (Miyazaki et al., 1988).

Recent experiments have tried to duplicate the repetitive $[Ca^{2+}]_i$ oscillations that occur in fertilized oocytes to determine if the developmental rate of parthenogenetically

activated oocytes could be enhanced. Repeated application of two 1.8 $kV \cdot cm^{-1}$ electric pulses every 4 min for 1.5 h was used to induce a periodic rise in $[Ca^{2+}]_i$ within rabbit oocytes (Ozil, 1990). This treatment significantly enhanced in vitro development to the blastocyst stage as well as resulted in a high rate of post-implantation development (66%) to day 10-11. These results indicate repetitive electric pulses, and therefore repetitive $[Ca^{2+}]_i$ oscillations, enhance parthenogenetic development in the rabbit. This is supported by reports in cartilage and osteoblast-like (MC3T3-E1) cells that indicate oscillating fields stimulate DNA synthesis in vitro. Oscillating electric fields (1.16 $kV \cdot cm^{-1}$, 5 Hz) applied to day-16 chick cartilage cells stimulated ^3H-thymidine incorporation (Rodan et al., 1978). DNA synthesis was also enhanced in osteoblast-like cells (MC3t3-E1) after 3.19 $kV \cdot cm^{-1}$, 3 msec, 10 Hz pulses were applied for durations ranging from 5 min to 20 h (Ozawa et al., 1989). Both reports concluded that DNA synthesis was stimulated, at least partially, by electric field induced fluctuations in $[Ca^{2+}]_i$.

Data presented here supports the model of CICR (Igusa and Miyazaki, 1983; Berridge, 1988) since IP3 electroporated into the cell and maintained at high levels generate transient $[Ca^{2+}]_i$ oscillations (Miyazaki, 1991). We infer that the intracellular concentration of IP3 was maintained at an elevated level since previous experiments using the same electropulse procedure showed murine oocyte pores to remain open for at least 5 min after treatment (Chapter III).

Igusa and Miyazaki (1983) reported that when IP3 was continually infused into the cytosol of hamster oocytes, $[Ca^{2+}]_i$ oscillations would cease within 2 min after the introduction of Ca^{2+}-free medium. Hence, influx of external Ca^{2+} into the cytosol

appears to be required to replenish internal Ca^{2+} stores once they have been depleted by multiple Ca^{2+} releases. Data obtained in the mouse (figure 5) indicates that repeated release of intracellular store Ca^{2+} is observed for a minimum of 20 min after oocytes are subjected to an electropulse in Ca^{2+}-free PBS containing 25 μm IP3. However, as reported in the hamster (Igusa and Miyazaki, 1983), the interval between oscillations was progressively longer in most oocytes observed.

In the mouse, ETOH parthenogenetically activates approximately 80-100% of oocytes placed in a 7% v/v concentration of ETOH in medium (Kaufman, 1982). In vitro development to the blastocyst stage is also consistently as good or better that other activation procedures (Kaufman, 1983). Colonna et al., (1989) reported a 6% ETOH treatment elicited a massive influx of Ca^{2+} into the murine oocyte during the first 5 min after activation and remained high for at least 15-20 min. When oocytes were pretreated with ETOH and then cultured for 5-60 min in medium containing ^{45}Ca, a significant increase in membrane permeability to Ca^{2+} was observed over control oocytes. It was concluded that the rapid rise in $[Ca^{2+}]_i$ was due to an increase in oocyte membrane permeability to Ca^{2+}. Data presented here indicates that ETOH activation causes a dramatic release of $Ca^{2+}{}_i$ from internal stores as well (figure 6). There was no difference in the fluorescent intensity between oocytes treated in ETOH-PBS or ETOH-Ca^{2+}-free PBS suggesting that the immediate rise in $[Ca^{2+}]_i$ was primarily due to release of intracellular stored Ca^{2+} and not primarily due to an increase in oocyte membrane permeability to extracellular Ca^{2+}.

Electroporation of IP3 may be useful in experiments involving electric pulse induced parthenogenetic activation. Artificially induced repetitive $[Ca^{2+}]_i$ oscillations

may enhance development of parthenogenetically activated oocytes. Electric pulse generated $[Ca^{2+}]_i$ oscillations may aid in the synthesis of DNA and enhance reprogramming of transferred nuclei. Electroporation of IP3 may experimentally provide $[Ca^{2+}]_i$ oscillations that coincide with an increase in protein and DNA synthesis.

In conclusion, a dramatic increase in $[Ca^{2+}]_i$ resulted from the release of Ca^{2+} from intracellular stores when murine oocytes were subjected to 7% ETOH in PBS or Ca^{2+}-free PBS. In addition, electroporation of IP3 into the cytosol elicits a dramatic rise in $[Ca^{2+}]_i$ in murine oocytes pulsed in Ca^{2+}-free medium. Periodic $[Ca^{2+}]_i$ oscillations were observed to occur for a minimum of 20 min after influx of IP3. The ability to consistently produce repetitive $[Ca^{2+}]_i$ oscillations may aid in the study of post fertilization development and cell cycle events. Current studies are being conducted to determine if IP3 can be used to enhance rate of electric pulse induced parthenogenesis and subsequent development.

CHAPTER VI

CONCLUSIONS

Upon fertilization, mammalian oocytes exhibit a rise in $[Ca^{2+}]_i$ which corresponds with activation of cellular events leading to the resumption of meiosis and mitosis. Activation of mammalian oocytes can be induced by subjecting oocytes to a DC electrofusion pulse. Electrofusion pulse induced activation in murine oocytes can be directly related to the elevated levels of Ca^{2+} ions in the fusion medium. Figures 1 and 2 illustrate a dramatic increase in $[Ca^{2+}]_i$ upon application of a fusion pulse in nonelectrolyte (0.3 M mannitol) and electrolyte (PBS) fusion media containing 0.09 mM Ca^{2+}. This increase in $[Ca^{2+}]_i$ had a direct affect on oocyte activation as a significantly higher rate of activation was observed when oocytes were pulsed in medium containing 0.9 mM Ca^{2+} (table 2). In addition, when IP3 was added to Ca^{2+}-free PBS fusion medium, an initial increase in $[Ca^{2+}]_i$ was observed followed by a series of periodic $[Ca^{2+}]_i$ oscillations (figure 5). These $[Ca^{2+}]_i$ oscillations appear to mimic those observed in recently fertilized oocytes.

Membrane permeability to extracellular Ca^{2+} was shown to remain elevated after fusion pulse treatment (figure 3). A significant increase in $[Ca^{2+}]_i$ was observed when Ca^{2+} ions were added to Ca^{2+}-free fusion medium 5 min after application of a fusion pulse. Assuming membrane permeability to extracellular Ca^{2+} remained elevated in pulsed oocytes used for assessment of activation, a comparable change in $[Ca^{2+}]_i$ should have occurred when the oocytes were removed from the fusion medium and placed into Whitten's medium 2 min after fusion pulse. However, the possible rise in $[Ca^{2+}]_i$ 2 min after fusion pulse did not effect rate of activation to the extent as was

observed when oocytes were pulsed in medium containing 0.9 mM Ca^{2+}. It is possible that the effect of an increase in $[Ca^{2+}]_i$ on oocyte activation may be time dependant.

We have shown that PBS can be effectively used to fuse murine pronuclear stage transplantation embryos with no difference in embryonic viability in vitro compared to nonelectrolyte (0.3 M mannitol) fusion medium. It may be beneficial to fuse nuclear transfer embryos in the same ionic strength medium as is used during micromanipulation to possibly decrease ionic stress and subsequent cell lysis that has been observed in nonelectrolyte fusion medium.

These findings have important implications in increasing the efficiency of mammalian nuclear transplantation. We have shown a 2.5 fold increase in the rate of activation in oocytes pulsed in fusion medium containing elevated levels of Ca^{2+}. Significant increases in development of nuclear transfer embryos should be realized through implementation of these procedures. In addition, future experiments will determine if the periodic oscillations in $[Ca^{2+}]_i$ induced by IP3 can initiate and mimic developmental events that occur at fertilization. Additional experiments designed to study changes in $[Ca^{2+}]_i$ and its regulatory role in initiating resumption of meiotic and mitotic events may lead to a better understanding of early mammalian development as well as an increase in efficiency of nuclear transplantation.

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VITA

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Lee F. Rickords

Major Field: Animal Science

Title of Dissertation: Nuclear Transplantation: Intracellular release and extracellular influx of calcium in response to electrofusion pulse and its effect on murine oocyte activation and embryonic development.

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

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